Genetic analyses of pre-meiotic DNA replication in *Saccharomyces cerevisiae*

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Abstract

Precise and complete replication of the genome is essential for a cell. Chromosome replication follows a defined temporal order, depending on the efficiency and timing of the replication origins. However, the mechanism regulating origin activity has not been properly explained to date.

Yeast replication origins are very well characterized and well studied. Genome wide replication in yeast was detailed through deep sequencing in various studies. In yeast, there are multiple replication origins for the complete replication of the genome. In *Saccharomyces cerevisiae,* there are ~400 replication origins, which are also referred to as Autonomously Replicating Sequences. Replication origins have varied levels of activity and varying times of activation. There are many lines of evidences, which suggest that the origins function differently in mitotic and meiotic cell cycles. It was thought that same origins function both during mitosis and meiosis. However, there is a difference in the replication timings of both the cell cycles, the reason for which is not known. Meiotic cell cycle is longer than the mitotic cell cycle. By using the plasmid-based assays, specific origins were selected and origin activity was analyzed during mitosis and meiosis to see if individual origins show any differences in origin activity. For all the origins tested, the meiotic activity was found to be less than the mitotic activity, which provides a possible explanation for a longer pre-meiotic S phase.

Most of the confirmed yeast replication origins are present in the intergenic regions of the chromosome. Due to the presence of majority of replication origins in the intergenic regions and not on the genes, it was thought that the gene transcription might be detrimental to origin activity, hence not supporting the existence of an origin on a gene. Careful analysis of genome wide replication data along with plasmid based ARS assays confirmed that a few replication origins are present within genes. Assays were preformed to study the relation between transcription and origin activity both during mitosis and meiosis. Mitotic origin activity was shown to have no known affect from gene transcription. However, due to some unknown technical faults or other reasons, assays to find out transcription and meiotic activity were not successful.

I would like to dedicate my thesis to my beloved parents and all my teachers

Acknowledgements

I would like to express my deep gratitude and thanks to my PhD supervisor Conrad Nieduszynski, who has supported me through my PhD, for his constant support, enthusiastic encouragement and motivation. I attribute the level of my research to his encouragement and effort. One simply could not wish for a better, friendly supervisor. Very big thanks to the present and past lab members for their scientific help, especially Michelle and Carolin for their encouragement and friendship. Thanks to Dr. Ed Louis for all the useful scientific advice and guidance. I would like to thank Dr. Bill Wickstead, Dr. Allers Thorsten for the advice and assistance. I would also like to extend my thanks to the technicians of the laboratory in the department for their help in offering me the resources for my research. I also take this opportunity to express gratitude to the University of Nottingham, for offering me the scholarship to pursue my research.

I would like to thank my husband for his support and my little princess for being my inspiration and relaxation during tough times. A special thanks to my close friends and family for being there for me. Lastly, I would like to thank the three most important people of my life, my parents and my brother, who have always been there by my side right from the beginning offering constant love, strength and support. I would never be the person I am without their unconditional love and support.

Abbreviations

ACS	ARS consensus sequence
ARS	Autonomously replicating sequence
bp	base pair
BSA	Bovine serum albumin
CDK	Cyclin-dependant kinases
CEN	Centromere
ChIP	Chromatin Immunoprecipitation
Chr	Chromosome
DDK	Dbf4-dependant kinase
dNTP	Deoxy nucleoside triphosphate
DSB	Double strand breaks
dsDNA	Double stranded DNA
EDTA	Ethylene diamine tetraacetic acid
FACS	Flourescence-activated cell sorting
GFP	Green fluorescent protein
kb	Kilo basepair
OD_{600}	Optical density at 600nm
ORC	Origin recognition complex
ORF	Open reading frame
OriDB	DNA replication origin database (www.cerevisiae.oridb.org/)
PEG	Polyethylene glycol
Pol	Polymerase
pre-RC	Pre-replication complex
SDS	Sodium dodecyl sulphate
SGRP	Saccharomyces Genome Resequencing Project
ssDNA	Single stranded DNA
wt	wild type
YPD	Yeast-peptone-dextrose media

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1 Introduction

1.1 *Saccharomyces cerevisie* as an experimental organism

Yeast, *Saccharomyces cerevisiae (S. cerevisiae)* is one of the most commonly used eukaryotic model organisms. Yeast is a unicellular organism and appears to have very little in common with human beings, however the most important feature is that yeast is a eukaryotic organism, containing nucleus with chromosomes similar to the human cells. Another similarity of yeast and human cells is that cell division is regulated in a similar way in both organisms. About 20% of human disease genes have counterparts in yeast. This suggests that some diseases results from the disruption of basic cellular processes like the cell division, control of gene expression or DNA repair. This relativity suggests that yeast can be used for testing the functional relationships involving these particular genes and to test new drugs. A yeast mutant that has lost the functional equivalent of a human disease gene can be screened with thousands of potential drugs in order to identify compounds that restore normal function to the yeast cell. These compounds, or molecules like them, might also be useful in humans. Moreover gene manipulation is easy and cheap in yeast compared to other eukaryotes.

Other advantages of using the yeast genetic system include the non-pathogenic nature of the organism, ease of handling it with fewer precautions. The very fast growth rate of the yeast makes it easy for the researchers minimizing the waiting time. Yeast has a highly versatile DNA transformation system. Yeast can be stably maintained in both diploid and haploid states, thereby facilitating genetic analysis. The stable existence of yeast in haploid form allows the ready detection of recessive mutations, thereby providing an opportunity of applying powerful genetic applications. Active recombination machinery allows targeted integration and gene manipulation. There are various sophisticated techniques that allow targeted manipulation of the genome, like gene deletions etc. The relatively small genome size of yeast *Saccharomyces cerevisiae* (12 megabases), which was completely sequenced, makes it a powerful tool for genetic analyses (Goffeau et al., 1996) (Mewes et al., 1997). Yeast has also been successfully used as a model studying human diseases (Gammie et al., 2007).

Yeast reproduces both sexually and asexually, however asexual reproduction by budding is the most common mode of reproduction in yeast. The parent cell buds off the daughter cell with its nucleus splitting apart. Budding yeast lives either as diploid or haploid. In either case reproduction is by forming buds via mitosis. Haploid cells are either ‘a’ or ‘α’

(alpha) mating type, which can be determined by gene expression at an active mating type locus. Diploid cells have more resistance to harsh environmental conditions. Sporulation is induced only as a result of these stressful environmental conditions. Under starvation, the cells undergo meiosis producing four spores in an ascus. Under suitable conditions, the spores germinated to produce four haploid yeast cells (2a and 2α) (Figure1).

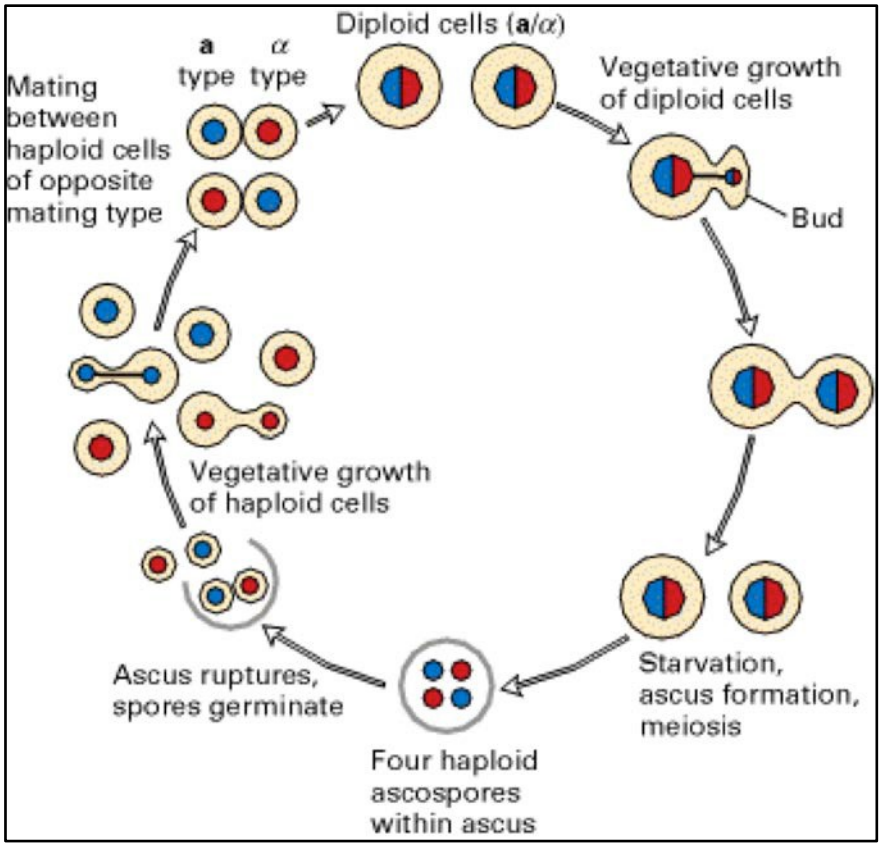


Figure 1: Life Cycle of *S cerevisiae*. Two haploid cells that differ in mating type, called a and α, can mate to form a diploid a/α cell, which multiplies by budding. Under starvation conditions, diploid cells undergo meiosis, forming haploid ascospores. Rupture of an ascus releases four haploid spores, which can germinate into haploid cells. Once each generation a haploid cell is converted to the opposite mating type (Lodish H, 2000).

1.2 Yeast Genome

Yeast was the first eukaryotic organism whose genome was completely sequenced (Dujon, 1996) (Goffeau et al., 1996). The yeast genome consists of 16 chromosomes in haploid form, varying in size from 250 kb to 2500 kb. The genome constitutes 12,052 kb with 6,183

open reading frames (ORF's) of over 100 amino acids, among which approximately 5,800 were predicted to correlate with actual protein coding genes. The yeast genome is highly compact when compared to the genome of multicellular organisms, with 72% of the genome covered with genes. Yeast genes have an average size of 1.45 kb or an average of 483 codons, ranging from 40 to 4,910 codons. The DNA sequence reveals that yeast has 262 tRNA genes; 80 of them having introns. The circular plasmids present in almost all the strains of *Saccharomyces cerevisiae* have the ability to replicate on their own (Velmurugan et al., 2000).

The functional elements of yeast chromosome are the centromeres, origins of replication and the telomeres. The centromere is the site of kinetochore formation and plays a vital role in the correct segregation of chromosomes during mitosis and meiosis. The yeast centromeric DNA sequence is compact when compared to higher eukaryotes, extending to only 200bp. Multiple replication origins are present on all chromosomes. The function of origins is explained in detail in section 1.4.1. The ends of the chromosome are flanked by telomeric and sub telomeric elements (Louis, 1995). Telomeres function to protect the chromosome ends from fusion, promote chromosomal segregation, maintains chromosomal stability and to prevent erosion of the chromosome end due to the end "replication problem" (Louis, 1995). The length of telomeres varies greatly between species, ranging from approximately 300 base pairs in yeast to many kilobases in humans. In vertebrates, the sequence of nucleotides in telomeres is TTAGGG and this sequence of nucleotides is repeated approximately 2,500 times in humans.

Yeast genome consists of transposable Ty elements, which appear to resemble a primitive retrovirus. A haploid yeast genome consists of around 35 copies of Ty elements. Ty elements are 6kb in length and consists of a central coding region flanked by about 340bp sequence called long terminal repeats (LTRs) at both ends in a direct orientation. Ty elements transpose by a mechanism similar to retrovirus reverse transcription and integration {Xu, 1987 #1}.

1.3 Cell Cycle

1.3.1 Mitosis

In eukaryotes, the cell cycle is divided into two phases, interphase and mitosis. During interphase, the cell grows by accumulating all the nutrients required for duplicating the DNA and other cellular components (e.g. mitochondria). During mitosis, the cell splits into two different cells called the daughter cells. The last phase is cytokinesis where the daughter cell gets completely separated by the division of the cytoplasm. Interphase is divided into three

different phases, G1 phase, S phase and G2 phase (Figure 2). The G1 phase is the pre-synthesis gap phase and cells leave the G1 phase only after reaching a critical size. The cells increase in their size during the G1 phase, which is also known as the growth phase. In this phase various enzymes are synthesized which are essential during the DNA replication. S phase is the synthesis phase, during which DNA replication takes place producing two identical sets of chromosomes. DNA replication is explained in detail in section 1.4. G2 phase is the post gap phase, which is the gap point of the cell between synthesis of DNA and mitosis. The cells continue to grow in the G2 phase until they enter the mitotic phase. Mitosis consists of several phases known as prophase, metaphase, anaphase, telophase and is completed with cytokinesis. The cells stop growing during the mitotic phase and start dividing to form two daughter cells. Mitosis is highly complex and tightly regulated. During the process of mitosis, the chromosome pairs condense and join to fibers, which pull the sister chromatids to the two extreme ends of the cell. The cell then divides completely in cytokinesis, producing two similar daughter cells.

The G0 phase or resting phase is a period in the cell cycle in which cell exist in quiescent state. G0 phase is considered either as an extended G1 phase where the cell neither divides or in a preparation state for dividing or a distinct quiescent stage that occurs outside the cell cycle. The commitment to replicate the genome and thus to enter mitosis is made at the 'Restriction Point' called START in budding yeast, during G1. It is influenced by various factors like cell size, growth factor, nutrients and DNA damage. If conditions are favorable, cells divide as often as possible, when there are sufficient nutrients around. However in the opposite conditions, diploid yeast cells enter meiosis. If the conditions are very poor and the cells are threatened to die due to starvation or other factors, they induce meiosis. Thus, the yeast cells hand their genetic material to the next generation, depending on their nutrient and environmental conditions. G_0 phase of the cell cycle is the resting phase in which cells are present in a quiescent state During the cell cycle, cyclins play a role in regulating the entry of cells in to different phases of cell cycle. Activity of Cln3/Cdc28 during the cell cycle is required for the cells to pass ‘start’, which is the commitment point to enter S phase. Cln1 and Cln2 interact with Cdc28 to promote the activation of cyclin associated CDK’s, which drives replication and mitotic entry. Specifically, association of Cdc28 with Clb1 and Clb2 promotes the entry of cells in to mitosis.

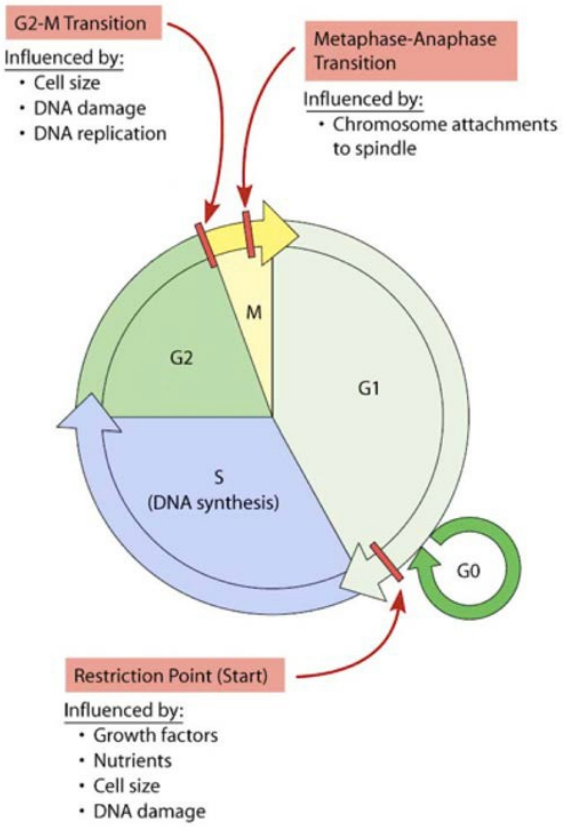


Figure 2: Eukaryotic cell cycle. Cell cycle highlighting influences on phase transition points. Before each cell division chromosomes are duplicated during the S-phase. Once the genome has been completely replicated daughter cells bud off in late mitosis (M- phase). During G1 (gap phase 1) cell growth occurs and cells either proceed through the cell cycle again or enter a state of quiescence (G0) (Becker et al., 2009).

1.3.2 Meiosis

After the daughter cells get completely divided at the end of mitotic division, the diploid cells can either enter into the mitotic cell cycle under favorable conditions or into a meiotic cycle under unfavorable conditions such as environmental factors or the lack of nutrients. Under starvation, the diploids enter meiosis, sporulate and finally propagate into 4 individual haploid spores (Figure 3).

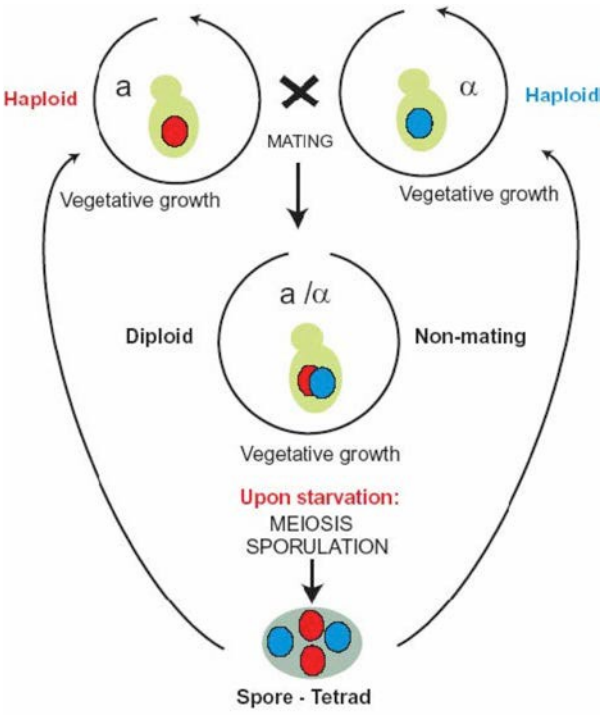


Figure 3: Meiosis The diploid cells, under starvation undergo meiosis, sporulate and produce four haploid spores.

In meiosis, chromosomes undergo recombination, where gene rearrangements take place producing a varied genetic combination in the resulting gametes. Recombination of chromosomes plays an important role in meiosis for proper chromosome segregation. Meiotic recombination starts off with double strand breaks (DSB's), which are introduced by a specific protein called Spo11 protein. Meiotic recombination results in haploid gametes, which contain recombinant chromosomes, with parts derived from both the parents. Recombination brings the homologous chromatids together. During meiosis I, sister chromatids acts as a single unit, the homologous kinetochore pairs that are attached to the spindle poles, are pulled towards the opposite poles. However, unless the reciprocal exchange of the sister chromatids occur, disjunction and segregation to opposite poles is opposed by the cohesion between sister chromatids. Thus, loss of cohesion between the sister chromatids along the chromosome arms is very much essential for the precise segregation of chromosomes during meiosis I. However, cohesion between the centromeres of sister chromatids still persists, which is later used in the alignment of sister chromatids during the metaphase stage of meiosis II. Hence, the difference in timing profile of cohesion loss between the sister chromatids is a crucial aspect.

Crossover and non-crossover recombination events occur during meiosis. During meiosis, crossovers occur at a high level, but the level of noncrossover recombinants is even higher

{Storlazzi, 1995 #2}. In eukaryotes, recombination during meiosis is facilitated by chromosomal crossover.

1.3.2.1 Meiotic transcriptional regulation

The mating type of the strain and the nutritional conditions regulates the process of meiosis and its initiation in *Saccharromyces cerevisiae*. Meiosis occurs in Matα/Mata cells, when the cells enter the nitrogen-depleting medium, in the presence of a carbon source and absence of glucose. These entry condition results in the expression of Ime1, which is a key regulator of meiosis. Further, Ime1 on association with a DNA binding protein, Ume6 encodes a transcriptional activator, which is recruited to the early expressed meiosis specific genes. Expression of early genes promotes entry into meiotic cycle, as they include genes required for pre-meiotic DNA synthesis, meiotic recombination and synapsis of homologous chromosomes. Ndt80, a transcriptional activator and Ime2, a CDK homologue are the two early meiotic specific genes, which are required for the transcription of mid-meiotic specific genes, which are responsible for spore formation and nuclear division. After the spore formation, maturation of the spore depends on late genes, whose expression is indirectly dependent on Ime1, Ime2 and Ndt80. Phosphorylation of Ime1 by Ime2 results in it degradation followed by subsequent shutting down of meiotic transcriptional cascade (Kassir et al., 2003). The initiation and progression stages of meiotic cell cycle are governed by a transcriptional cascade consisting of programmed and temporal expression of meiosis-specific genes (Primig et al., 2000). *IME1* is a key regulatory gene, which is required for meiotic cell cycle entry and the transcription of all meiosis specific genes (Smith and Mitchell, 1989) (Sagee et al., 1998) (Figure 4).

Transcriptional timing and meiotic function are also co-related. The transcription of each of the genes expressed during different phases of meiosis are linked to various functions prior to, during and after pre-meiotic DNA replication. Transcription of early meiotic genes (EMG) like *HOP1* and *DMC1* is induced prior to pre-meiotic DNA replication, which is required for pairing of homologous chromosomes and meiotic recombination functionalities respectively. Induction of cell division cycle (CDC) genes (*POL1*) is essential during the process of pre-meiotic DNA replication and the process of transcription of CDC genes takes place at the same time of pre-meiotic DNA replication. Middle meiotic genes (MMG) like *CLB1, 2, 3, CDC26* are induced and are involved in nuclear division; they are induced after DNA replication and before the first nuclear division. The late meiotic genes (LMG) are induced only after the

completion of nuclear divisions and are involved in spore formation and maturation. However, some genes do not follow the correlation of transcriptional timing and meiotic functionality, for example Middle genes *CLB5* and *CLB6* are essential for pre-meiotic DNA replication (Kassir et al., 2003).

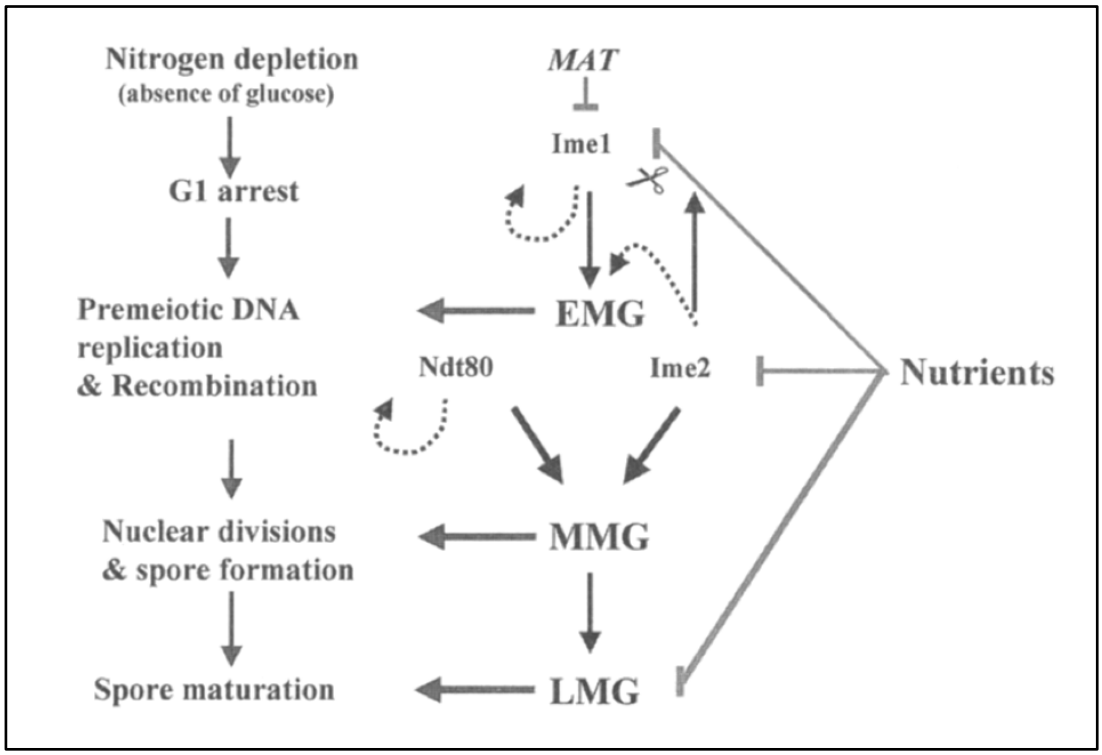


Figure 4: A transcriptional cascade governs initiation of meiosis Meiotic entry of Mata/Matα in the absence of nitrogen and glucose, leads to G1 arrest and Ime1 activation. Transcription of early meiosis specific genes (EMG) is triggered by IME1 encoding a transcriptional activator. Ndt80 and Ime2 are essential for the transcription of middle meiotic genes (MMG). Ime1 and Ime2 are responsible for the transcription of late meiotic genes (LMG), which are required for spore maturation **(Kassir et al., 2003).**

1.3.3 Regulation of Cell Cycle

The cell cycle events like mitosis, cytokinesis, DNA replication should be executed in a correct order so that the cell's genome can be passed intact to the next generation. Many proteins regulate the cell cycle, mainly cyclins and their regulators (inhibitors, activators). The main regulator of the budding yeast cell cycle is the cyclin-dependent protein kinase (Cdc28),

which is encoded by the gene *CDC28*. The critical regulatory step in the cell cycle is called 'START' (Figure 5).

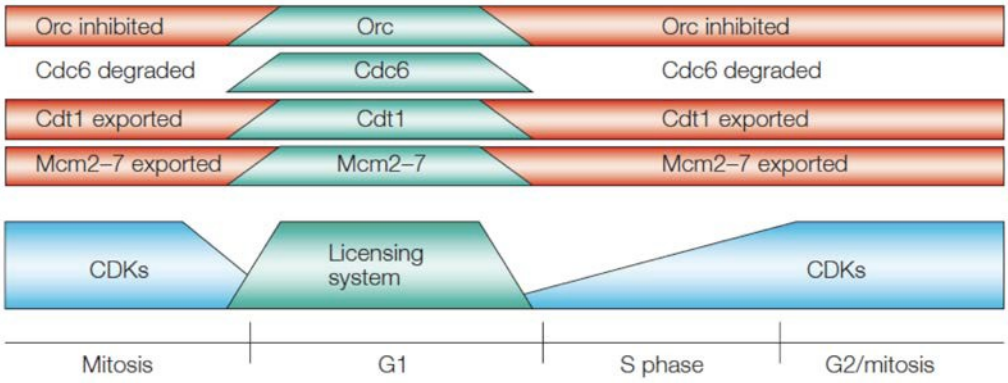


Figure 5: Cell-cycle regulation of the licensing system in *Saccharomyces cerevisiae*. The activity of components of the licensing system during the cell cycle of yeast is shown. In the lower part of the figure, the licensing system is shown. This is active (green) only in G1. In yeast, licensing is inhibited at other times by cyclin-dependent kinases (CDKs; blue). Above this, the activity of different pre-replicative complex components (origin recognition complex (ORC), Cdc6, Cdt1 and minichromosome maintenance (Mcm2–7 proteins) are shown. Green indicates active proteins; red indicates inactive/degraded or exported proteins (Blow and Dutta, 2005).

1.4 DNA replication

Complete and error free replication of the whole genome during a cell cycle is vital for genomic integrity and is hence essential for life. DNA replication is similar in all organisms. In prokaryotes, replication starts from a single locus. The process of DNA synthesis is not restricted to any particular time point and takes place through out the cell cycle. Therefore, in a single cell, there will be multiple rounds of ongoing replication. However in eukaryotes, the genome is larger than the prokaryotes and DNA replication is restricted to a particular stage in the cell cycle (the S phase). For the complete replication of the entire genome in a particular limited time, initiation of replication takes place at multiple regions, called the replication origins, along each chromosome. The replication origins must be well regulated to ensure that each origin activates only once in each cell cycle and that there is an appropriate distribution of active origins.

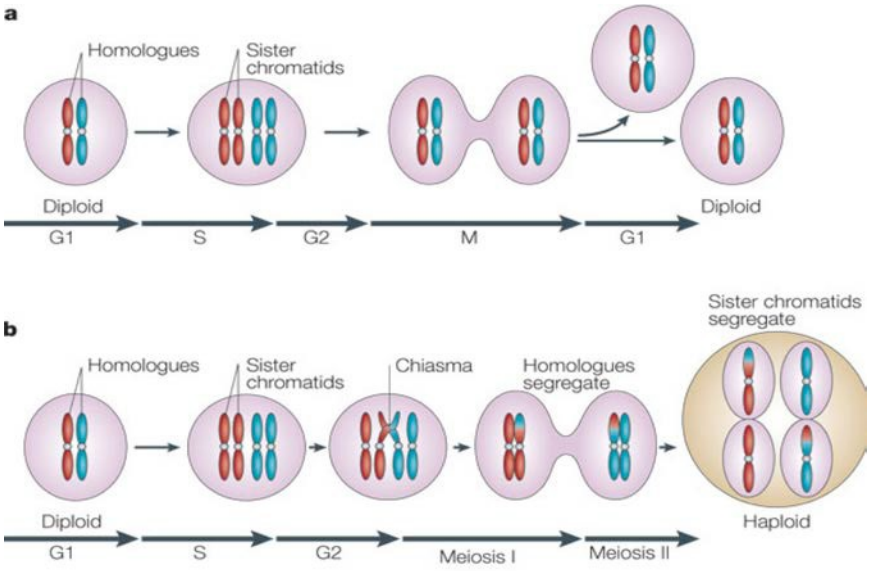


Figure 6: The mitotic and meiotic cell cycles a) In mitosis, diploid cells replicate chromosomes during S phase and segregate sister chromatids during M phase, so that diploid daughter cells are produced. b) In meiosis, two chromosome-segregation phases, meiosis I and meiosis II, follow a single round of DNA replication during pre-meiotic S phase. During meiosis I, homologous chromosomes (shown in red and blue) are segregated to opposite poles. Sister chromatids segregate to opposite poles during meiosis II, which results in the formation of non-identical haploid gametes (Marston et al., 2004).

DNA replication takes place at a particular time phase called the S phase and for a limited period of time. Replication of DNA starts at the replication origins. At each origin, two replication forks are initiated with each fork facing away from the other. The replication forks move along the DNA while replicating during the process. In most cases, replication forks terminate only upon meeting mother fork from the opposite direction. For the replication to result in the correct duplication of the genome, the replication origins should never initiate replication forks on the DNA, which has already replicated in that cell cycle that is regulated by the replication licensing system. To replicate large chromosomes eukaryotes uses multiple replication origins varying from thousands in humans (Jeon *et al*., 2005) to a few hundred in yeast (Nieduszynski et al., 2007) and the origins are spaced 30-100kb apart. Replication origins fire at different points during the S phase creating a 'replication timing program' thereby different chromosomal DNA segments replicate at different times. However it is unclear which factors determine the location of origins and the time of origin firing.

1.4.1 Replication origins

Eukaryotic eukaryotic genomes contain significantly more origins, ranging from 400 in yeast to 30,000–50,000 in humans {Mechali, 2010 #1285}. Each budding yeast origin consists of a short (~11 bp) essential DNA sequence (called the ARS consensus sequence or ACS) that recruits replication proteins. In other eukaryotes, including humans, the DNA sequences at the replication origins vary. Despite this sequence variation, all the origins form a base for assembly of a group of proteins known collectively as the pre-replication complex (pre-RC). In metazoans, pre-RC formation is inhibited by the protein geminin, which binds to and inactivates Cdt1. Regulation of replication, prevents the DNA from being replicated more than once each cell cycle. In humans an origin of replication has been originally identified near the Lamin B2 gene on chromosome 19 and the ORC binding to it has extensively been studied {Falaschi, 1994 #1}.go

Replication origins in budding yeast contain conserved sequence elements (Deshpande and Newlon, 1996), which direct the recruitment of the origin recognition complex. However, all the sites that possess the conserved sequence elements may not function as replication origins and hence additional factors are also involved. Origins are often referred to as Autonomously replicating sequence (ARS) elements because they have the ability of high frequency transformation and extra chromosomal replication of plasmids in the yeast *Saccharomyces cerevisiae*. This characteristic of ARS is useful for identifying origins via the plasmid stability assay and ARS assay (Hsiao and Carbon, 1979) (Stinchcomb et al., 1979). Functional sequence elements such as ARS consensus elements (ACS) and B elements were identified within the origins (Figure 7). The ACS consists of an 11 bp motif, (A/T)TTTA(T/C)(A/G)TTT(A/T), that tolerates no more than two mismatches to stay functional (Bell and Dutta, 2002) (Fabiani et al.,1996). The ACS is required for the function of replication origins; and each ARS has an ACS. Furthermore most ACS are also phylogenetically conserved among the *sensu stricto Saccharomyces* species (Nieduszynski et al., 2007).

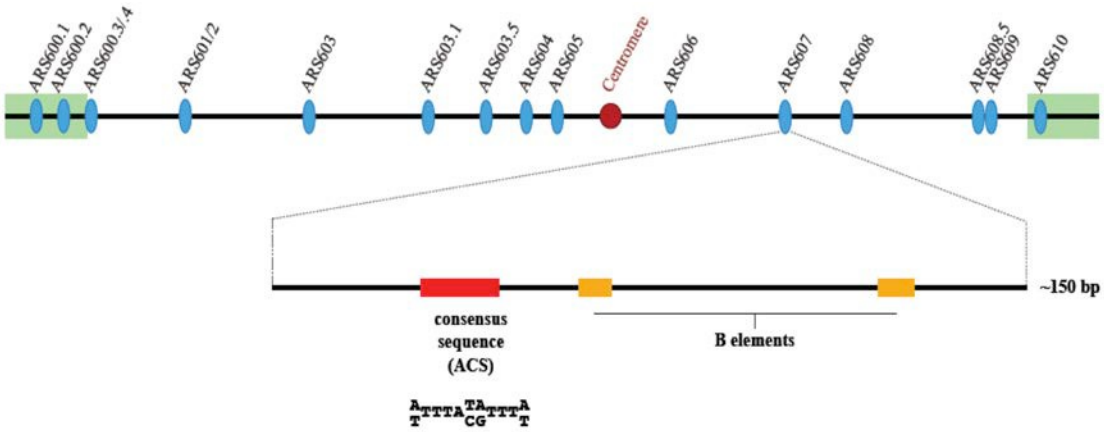


Figure 7: Substructure of replication origins in chromosome VI of Saccharomyces cerevisiae. In *S. cerevisiae* origins of replication span ~150 bp and every ARS contains the highly conserved 11 bp ACS. Additionally non-conserved B elements are usually found downstream of the ACS.

B elements (B1, B2, B3/B4), in addition to the ACS, also contribute to the origin acitivity, however they are not essential. B elements are non-conserved sequences 3' to the T-rich strand of the A element. B elements tend to be the DNA unwinding elements (DUE), with a reduced helical stability, facilitating unwinding of DNA during initiation (Lin and Kowalski, 1997) (Figure 8). The ACS and B element together recruit the ORC to ARS.

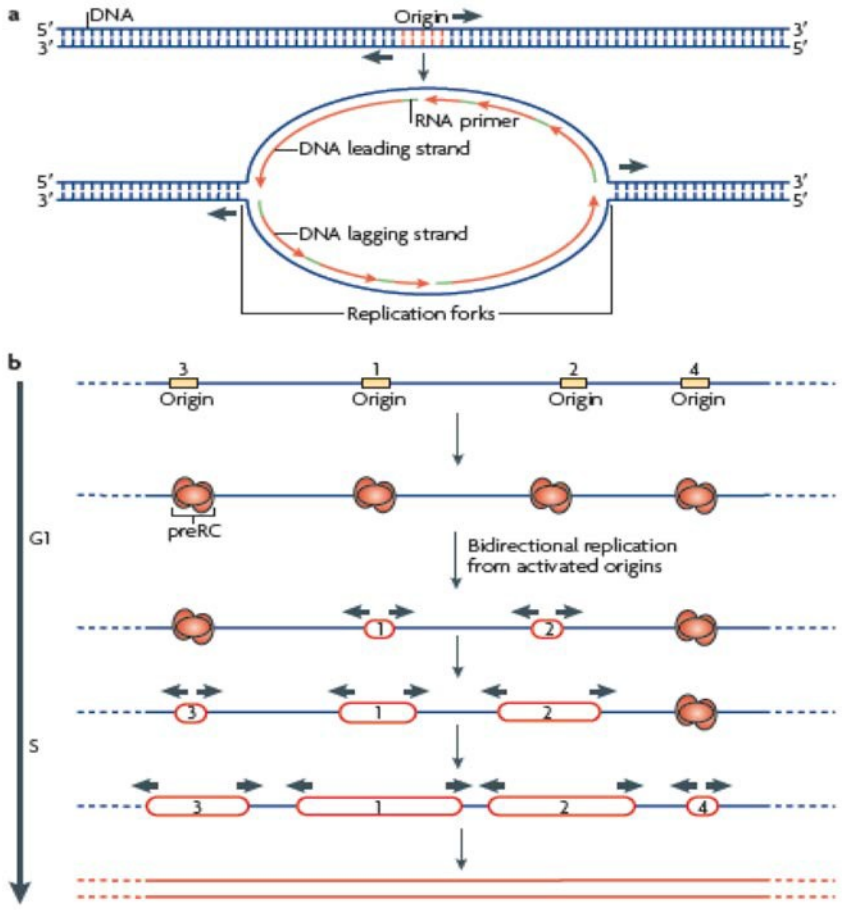


Figure 8: Activity of Replication origins. a) At each replication origin, DNA synthesis starts with short RNA primers that are synthesized by DNA polymerase-α. As DNA synthesis always occurs in the 5′–3′ direction, one strand of the DNA (the leading strand) will be synthesized continuously, whereas the other strand (the lagging strand) will be synthesized discontinuously by short RNA-primed DNA fragments. Two other DNA polymerases (δ _and ε) are recruited for the elongation of lagging and leading strands, respectively. b) Activation of replication origins during S phase. Pre-replication complexes (pre RCs) are assembled at replication origins during G1 phase. Activation of replication origins occurs throughout S phase, some during early (1 and 2), and some in mid (3) or late (4) S phase (Mechali, 2010).

1.4.2 Replication origin licensing and activation

To achieve precise duplication of chromosome during the S phase, replication origins have to be properly 'licensed' in order to 'fire' and subsequently produce divergent replication forks. Licensing occurs in late M phase and G1 phase. In budding yeast the origin recognition complex (ORC) is bound to the DNA all through the cell cycle and forms a core complex where the other protein components are recruited. ORC is highly conserved in all eukaryotes and consists of 6 subunits (Orc1-6). The initial step of the pre-replicative complex formation is the

recruitment of Cdc6 and Cdt1 by ORC (Figure 9). The ORC-Cdc6- Cdt1 complex recruits the hetero hexameric Mcm2-7 complex, thereby licensing the replication origin. A single ORC-Cdc6-Cdt1 complex can load many Mcm's in ATP dependent manner (Bowers *et al*., 2004). The resulting complex is called the pre replication complex (pre-RC) (Diffley *et al*., 1994) and the assembly of the pre RC takes place in the late M through G1 phase in the cell cycle. When the cell enters the stationary phase, the pre-RCs are removed from the origins of *S.cerevisiae* and reassemble upon re-entry into the cell cycle (Diffley et al., 1994).

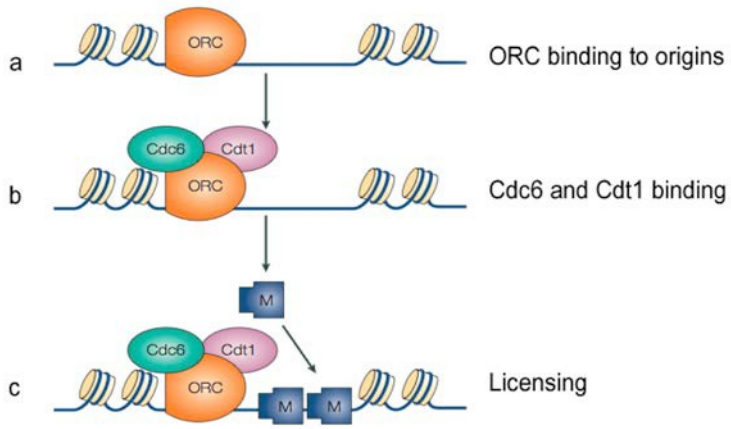


Figure 9: Stepwise assembly of pre-replicative complex proteins during origin licensing. a) The origin recognition complex (ORC) is first recruited to the replication origin. b) ORC recruits Cdc6 and Cdt1. c) ORC, Cdc6 and Cdt1 act together to load multiple minichromosome maintenance (Mcm)2–7 protein hexamers onto the origin, which licenses the DNA for replication (Aapro and Blower, 2005).

Cyclin-dependant kinase (CDK) activity promotes S-phase entry and origin activation while inhibiting pre-RC assembly. Hence licensing and activation of origins are separated temporarily (Figure 5). This is crucial to prevent several rounds of origin activation in a single cell, which would result in genomic instability. CDK and Cdc7- Dbf4 are essential for origin activation. The two protein kinases, CDK and DDK are essential for the initiation of chromosomal DNA replication (Tanaka and Araki, 2013). They are also involved in the activation of replisome. A passively replicated origin, which has not fired completely, is inactivated by the removal of pre-RC proteins, preventing endoreplication.

Immediately after unwinding of the DNA at the origins, the ssDNA binding protein RPA is loaded on both the DNA strands. These proteins stabilize the DNA single strands and are believed to be involved in the recruitment of DNA polymerase α, whose enzyme activity is required for initiation of DNA synthesis. Strand synthesis occurs exclusively in 5` to 3` direction

(Figure 10). Therefore, the leading strand (3` to 5` relative to direction of unwinding) replicates continuously. However, in contrast, the lagging strand replicates discontinuously by synthesizing 'Okazaki fragments', which are short RNA-DNA hybrid sequences (~150-200bp). RNA is removed by an endonuclease prior to ligation of these okazaki fragments. DNA replication terminates when the opposing replication forks converge and the newly synthesized strands are ligated. Mcm2-7 functions as a replicative helicase, which is required for the elongation process.

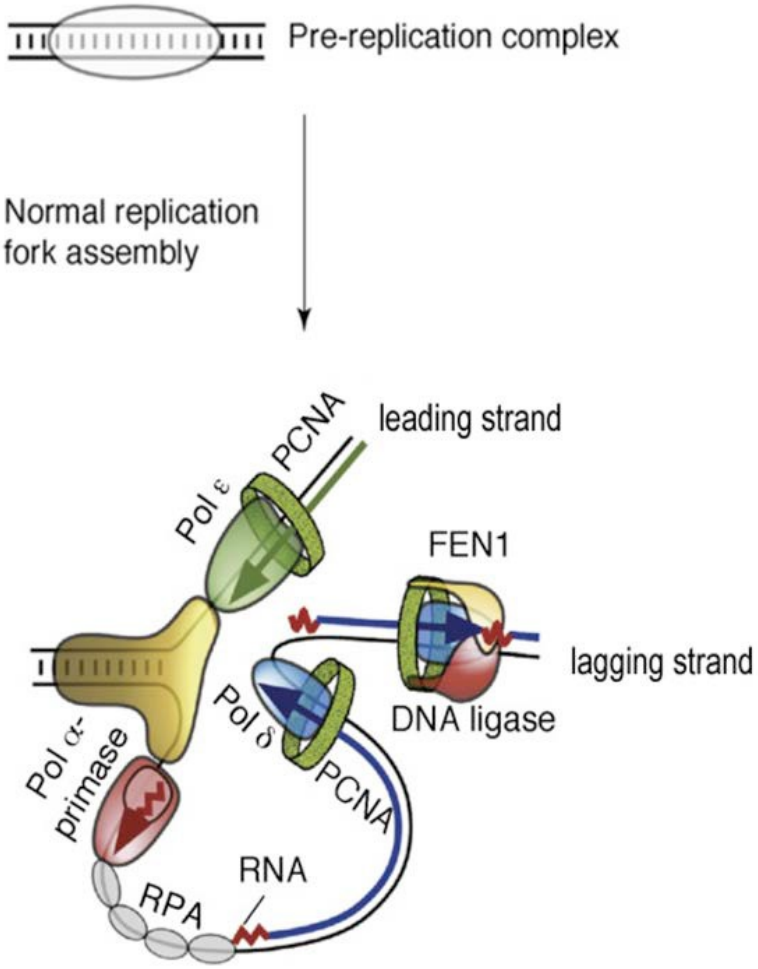


Figure 10: DNA replication forks in *Saccharomyces cerevisiae.* The model illustrates primary roles for Pol ε and Pol δ in leading- and lagging-strand replication, respectively. Other proteins shown include the Pol α-primase (red); the MCM helicase (yellow); the eukaryotic single-stranded-DNA-binding protein; replication protein A (RPA; gray); the sliding clamp proliferating cell nuclear antigen (PCNA; green); and the FEN1–DNA ligase complex (yellow-red) (Kunkel and Burgers, 2008).

1.4.3 Regulation of replication origin activity

Chromosome position influences the efficiency and timing of origin activation (Weinreich et al., 2004). Many observations in yeast show that origin activity is subjected to position effect, which resembles chromatin-mediated position effect in gene transcription. Some sequences, which provide ARS activity to plasmids, fail to function as chromosomal origins (Vujcic et al., 1999) (Newlon and Theis, 2002). Position effect also accounts for the activation of origins. Specialized chromatin structures are required for origin activation. It was shown that the telomere binding Ku protein complex (Ku) influences the late activation of telomere-proximal origins (Cosgrove et al., 2002). Replication timing correlates with chromatin structure and gene activity. Regions that replicate early are transcriptionally active and euchromatic, whereas late replicating regions are heterochromatic and transcriptionally silent (Weinreich et al., 2004) (Mechali, 2010) (Gilbert, 2002). Origin activity can be stimulated by transcription factors (Marahrens and Stillman, 1992) (Suter et al., 2004) (Danis et al., 2004). Transcription factors are thought to be involved in the recruitment of chromatin remodeling factors or increase origin accessibility to trans-acting factors (Flanagan and Peterson, 1999). Histone deacetylation suppresses or delays origin firing whereas histone acetylation stimulates or advances origin activity (Wyrick et al., 2001) (Aggarwal and Calvi, 2004) (Farkash-Amar et al., 2008). Chromatin structure may affect origin efficiency and timing. Studies show that histone acetylation is required for pre-RC assembly (Miotto and Struhl, 2007) and multiple acetylated lysines in histone H3 and H4 are essential for efficient origin activity.

1.4.4 DNA replication and transcription

Its been studied long about the relation of transcription with various other processes like replication, *ARS* function, centromere function etc. Investigators showed that transcription into a centromere can inhibit the *CEN* function *in vivo* (Snyder et al., 1988a). To function properly in yeast cells, both *CEN* and *ARS* should be protected from transcription. Conversely, it was shown that DNA segments flanking CEN4 protected *ARSI* from transcription and acted as strong transcription terminators in vivo. It has been reported that very high-level transcription across an origin reduces origin activity (Snyder et al., 1988a).

The precise relationship between origin function and transcription is uncertain. It was earlier shown that, in the case of *ARS121*, transcriptionally incompetent Abf1 protein fragments still stimulate replication, suggesting that DNA binding rather than transcription factor function is important for replication (Maine et al., 1984). One interpretation was that there is competition between the assembling pre-RC and the transcription apparatus, such that under normal dosage of Mcm2–7 proteins the origin is successfully licensed. However, under conditions of reduced Mcm2–7-protein function, the balance tips and transcription limits licensing and reduces the efficiency of origin firing (Nieduszynski et al., 2005). It was demonstrated that replication origin activity was negatively regulated through transcription, which interfered with the binding of ORC complex to the replication origin either directly by RNA polymerase or indirectly by transcription-associated events like changes in DNA topology or chromatin structure. A model was proposed to explain the inactivation of ARS activity by transcription (Mori and Shirahige, 2007a) (Figure 11).

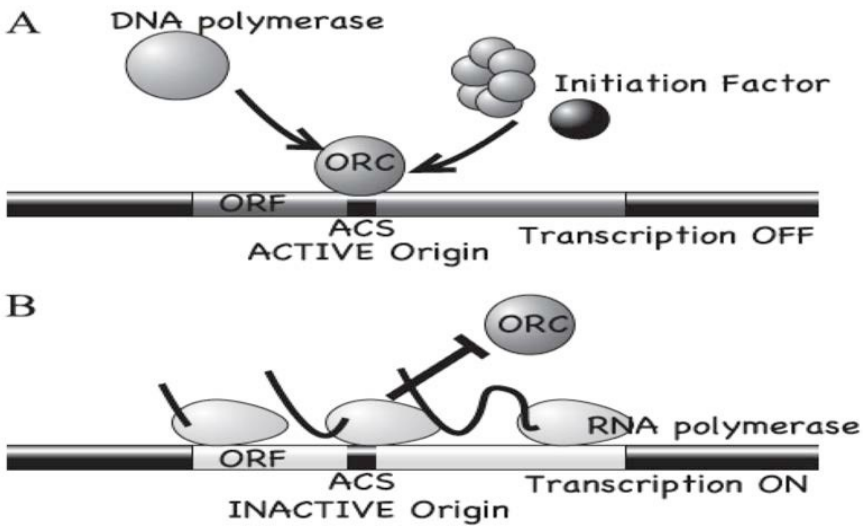


Figure 11: A scheme for the inverse correlation between origin activity and Transcription The initiation of DNA replication requires the stepwise association of several proteins at the origin of replication. When the initiator-binding site, the ACS, resides in an ORF, the origin can be activated in the absence of transcription. Switch from active to inactive state of replication origin is accompanied by removal of ORC complex from origin by transcription. In the absence of transcription, the origin is active and fired normally (A). When the transcription takes place, the origin function is prevented (inactive) as the result of dissociation of Orc1 from the origin probably either directly by the collision with RNA polymerase II or indirectly by transcription associated events like changes in DNA topology

1.4.5 Pre-meiotic DNA replication

In eukaryotes, mitotic and pre-meiotic S phase seems to be regulated differently. Pre-meiotic S phase is 2-3 times longer than the mitotic S phase taking approximately 65 minutes (Collins and Newlon, 1994a). Also, different genes are required for entry into both phases. The functions of *CDC4, CDC7, CDC28, CDC36* and *CDC39* gene products are required before DNA replication in a mitotic cell cycle, however in a meiotic cell cycle these gene products are required before meiosis I division and after DNA replication (Collins and Newlon, 1994b). Conversely, products of *SP07, SP08, SP09, ME II* and *ME12, 3* are essential for pre-meiotic DNA replication is not required for mitotic DNA replication. Comparative study on activity of origins during the pre-meiotic and mitotic S phase showed that *ARS605*, which is very efficient during mitotic S phase, is inhibited during the pre-meiotic S phase (Figure 12). *ARS605* is located in the ORF of *MSH4* gene, which is specifically transcribed during the early stage of meiosis. Systematic analysis between *ARS605* origin activity and *MSH4* transcription revealed that transcription of *MSH4* gene by RNA polymerase II inhibited the *ARS605* origin activity by removing ORC from *ARS605* during the pre-meiotic S phase (Mori and Shirahige, 2007b) (Figure 12). Determination of origin activity among the chromosome VI ARSs showed that the activity of all origins is similar except for *ARS605* in mitotic and premeiotic S phase (Mori and Shirahige, 2007b). The transcription of *MSH4* during the early meiosis (Ross-Macdonald and Roeder, 1994) (Chu et al., 1998) is assumed to be the cause of loss of origin activity in *ARS605*, because it is known that transcription from a strong inducible promoter interferes with origin functions on a plasmid (Snyder et al., 1988b).

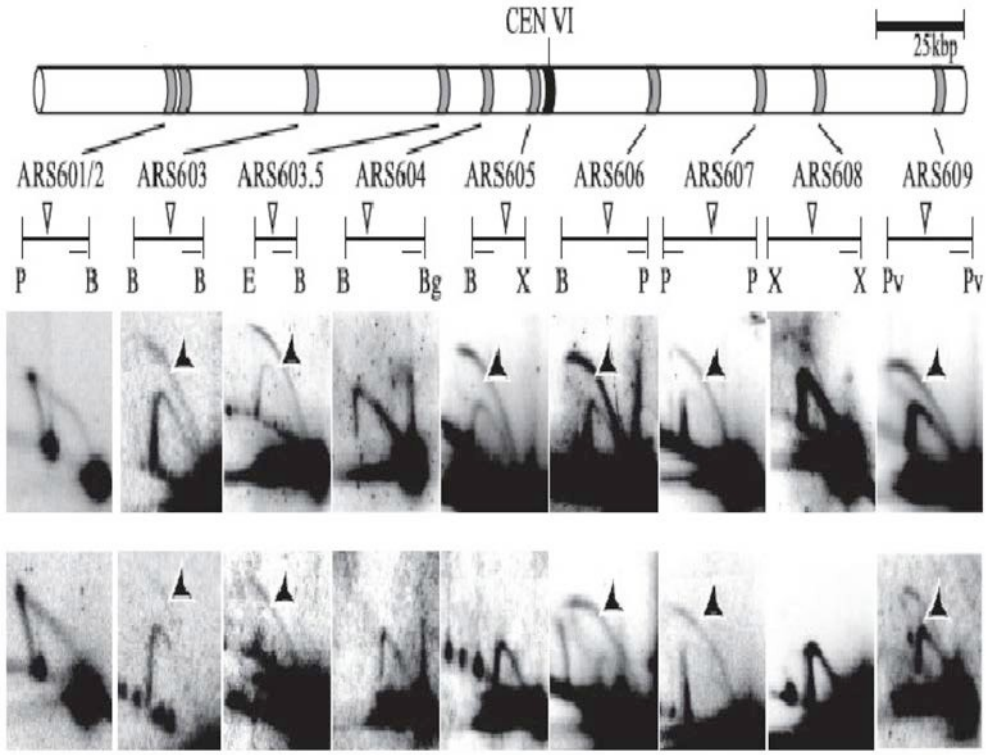


Figure 12: ARSs on the chromosome VI and their activity as DNA replication origins during the meiotic cell cycle Locations of ARSs on the physical map of the chromosome VI. The map is based on the published sequence of chromosome VI. The bar indicates 25 kbp. Bottom, the N/N two-dimensional gel electrophoresis of replication intermediates of nine chromosomal fragments containing ARSs from mitotic growing cells (upper row) and meiotic S-phase cells in the wild type (lower row). Arrowheads indicate bubble arcs for initiation/replication eye-form intermediates (Mori and Shirahige, 2007b).

The time to complete DNA replication during the synchronous pre-meiotic S phase culture was about 30% extended compared with the time in a synchronous mitotic S phase culture (Heichinger et al., 2006). The average efficiency of origin usage during pre-meiotic S phase was found to be only 15%, which is half when compared to that of mitotic S-phase. It was previously observed that Rec8 and Spo11 regulate the length of meiotic S phase (Cha et al., 2000), however other findings have shown that these proteins do not directly regulate the activation of early replication origins. Deletion of either of these proteins, Rec8 or Spo11 did not significantly alter the profile of pre-meiotic DNA replication in HU (Blitzblau et al., 2012).

1.4.6 Co-ordination of meiotic DNA replication with other meiotic events

A direct co-ordination was shown to exist between DNA replication and recombination initiation events. Meiotic DNA replication in *Saccharomyces cerevisiae* occurs 1.5 hours to 2.0 hours prior to recombination initiation. Blocking of meiotic replication prevented double strand break formation, whereas delay of replication on a particular chromosome segment delayed double strand break formation in that particular fragment. It was suggested that double strand break formation occurs as a part of a process initiated by DNA replication (Borde et al., 2000). Homologous recombination is activated during meiotic prophase, which is between the pre-meiotic S phase and the first meiotic division. Meiotic recombination plays a crucial role in ensuring faithful chromosome segregation during the first division (Yamada and Ohta, 2013).

Meiotic recombination initiation events through DNA double-strand break formation are temporally coordinated with DNA replication (Borde and Lichten, 2014). It was shown that this coordination requires the recruitment of Dbf4-dependant kinase to the replication fork. Chromosome replication initiates when two S phase kinases, cyclin-dependent kinase and Dbf4-dependent kinase (DDK) sequentially phosphorylate components of the replicative helicase (Tanaka and Araki, 2013). The mechanisms of DNA replication were as well found to be correlated and coordinated with the subsequent formation of double strand breaks (DSBs), which initiate recombination (Murakami and Keeney, 2014). It was shown in yeast that the replisome-associated components Tof1 and Csm3 physically associate with the Dbf4- dependent Cdc7 kinase (DDK) and recruit it to the replisome, where it phosphorylates the DSB-promoting factor Mer2 in the wake of the replication fork, synchronizing replication with an early prerequisite for DSB formation. Recruiting regulatory kinases to replisomes may be a general mechanism to ensure spatial and temporal coordination of replication with other chromosomal processes. The coordination between replication and DSB formation was proved by origin deletions. Deletion of a replication origin delays replication exclusively in the left arm of the chromosome, which results in a subsequent delay in DSB formation.

1.5 Objectives

The *Saccharomyces cerevisiae* genome contains nearly 400 autonomously replicating sequences (ARS), which function in binding ORC and other replication factors to the DNA. ARS consists of a 11bp ACS and different B elements. The main aim of my work focuses on studying replication origins and their activity particularly during meiosis. Replication origins and replication origin activity was very well studied in yeast, however replication and replication origin activity during meiosis is less understood.

It was long believed that there is a relation between transcription and origin activity. It was believed that transcription was thought to be detrimental to origin activity. Hence, the presence of gene was thought to be interfering with the presence of origin and its activity. However there was no clear confirmation of the affect of gene transcription on origin activity. I focused on finding out the relation between gene transcription and origin activity and if the proposed negative correlation between gene transcription and origin activity is true or not.

Majority of replication origins found in yeast till date were found to be present in between the genes. It was thought that the gene regions are detrimental for ARS sequences. I am interested in finding out why majority of origins are present in between the genes and if there are any additional origins present on genes. I aim to study the reason for the presence of origins in between the genes, if transcription really proves to be detrimental to origin sequences, as proposed earlier.

2 Materials and Methods

2.1 Chemicals and enzymes

Standard lab chemicals were purchased from Thermo Fisher Scientific, Roche and Sigma. New England Biolabs (NEB) supplied all the restriction enzymes. Taq DNA polymerase, including appropriate buffers were purchased from Bioline. Zymolase 20T and 100T were obtained from Seikagaku Corporation.

2.2 Growth media

All media was autoclaved at 115 °C for 10 minutes.

Low salt Lysogeny Broth (LB) media

Broth: 1% bacto tryptone, 0.5% yeast extract, 0.05% sodium chloride, pH adjusted to 7.0 with 1 M sodium hydroxide. For agar plates, 1.5% (4.5 g) agar were added to 300 ml broth. When appropriate, broth and agar plates were supplemented with 100 μg/ml ampicillin to select for the presence of the ampicillin resistance gene.

SOC broth

2% bacto tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate and 20 mM glucose.

Yeast-Peptone-Dextrose (YPD) media

Broth: 1% yeast extract, 2% peptone, 2% dextrose, 0.5% adenine. Broth was supplemented with 2% agar for solid media.

Antibiotic-containing media for yeast

YPD media was supplemented with the appropriated antibiotic to apply selection for a specific resistance gene (Table 1).

Table 1: Antibiotics used for selection of yeast strains

Antibiotic	Resistance gene	Selective concentration	Source
G-418	*KAN*	400 μg/ml	Gibco
Hygromycin B	*HPH*	300 μg/ml	InvivoGen

Minimal media

0.675% yeast nitrogen base minus amino acids (YNB), 2% dextrose, pH adjusted to 6.5 with 2.5 M sodium hydroxide. Broth was supplemented with 2% agar for solid media.

Synthetic dropout media

Minimal media supplemented with 875 mg/l of specific amino acid mixture. The nutrient mixtures lack specific supplement(s) to select for yeast strains with auxotrophic deficiencies. Media was named by specifying the amino acid(s) that are not in the mixture. Table 2 lists the amino acids and their concentration for a complete, non-dropout mixture.

Table 2: Composition of amino acids in synthetic complete media

Supplement (abbreviation used when dropped out)	Concentration
Argenine (ARG)	800 mg/l
Histidine (HIS	800 mg/l
Leucine (LEU)	800 mg/l
Tryptophan (TRP)	800 mg/l
Uracil (URA)	800 mg/l

Sporulation media (potassium acetate, KAC)

2% potassium acetate, 0.22% yeast extract, 0.5% dextrose, pH adjusted to 7.0 with 1 M hydrochloric acid or 2.5 M sodium hydroxide, then supplemented with 875 mg/l complete dropout powder. 2.5% agar was included for solid media.

2.3 Yeast strains, plasmids and oligonucleotides

The strains and plasmids used for the experiments described in this work are listed in Tables 3 and 4 respectively.

Table 3: Yeast strains used in this study

Strain	Relevant genotype	Description	Source
Derivates of *S. cerevisiae* SK1			
SMY010	*MATa/alpha; ho::LYS2/ho::LYS2 lys2/lys2 ura3/ura3*	Laboratory diploid SK1 strain	Ed Louis
SMY011	*MATa/alpha;* YCp*ARS605*	SMY010 transformed with pMSH001	this study
SMY015	*MATa/alpha;* YCp*ARS605*	SMY010 transformed with pMSH006	this study
SMY020	*MATa/alpha;* YCp*ARS607*	SMY010 transformed with pMSH007	this study
SMY023	*MATa/alpha;* YCp*ARS607*	SMY010 transformed with pCA011	this study
SMY027	*MATa/alpha;* YCp*ARS607*	SMY010 transformed with pSM005	this study
SMY031	*MATa/alpha;* YCp*ARS607*	SMY010 transformed with pSM006	this study
SMY035	*MATalpha*; *lys2 ura3 ho::LYS2 trp1:: hisG*	Wild type SK1 haploid strain	Ed Louis
SMY036	*MATa*; *lys2 ura3 ho:: LYS2 leu2-R his4-X arg4-Bgl*	Wild type SK1 haploid strain	Ed Louis
SMY037	*MATa/alpha; lys2/lys2 ura3/ura3 ho::LYS2/ho::LYS2 trp1::hisG/TRP1 LEU2/leu2-R HIS4/his4-X ARG4/arg4-Bgl*	Wild type diploid SK1 strain with selectable markers (SMY035 x SMY036)	this study
SMY041	*MATa/alpha;* YCp*ARS605*	SMY037 transformed with pMSH001	this study
SMY045	*MATa/alpha;* YCp*ARS605*	SMY037 transformed with pMSH006	this study
SMY049	*MATa/alpha;* YCp*ARS607*	SMY037 transformed with pMSH007	this study
SMY053	*MATa/alpha;* YCp*ARS607*	SMY037 transformed with pCA011	this study
SMY058	*MATa/alpha;* YCp*ARS607*	SMY037 transformed with pSM005	this study
SMY061	*MATa/alpha;* YCp*ARS607*	SMY037 transformed with pSM006	this study
SMY066	*MATa/alpha;* YCp*ARS605*	SMY037 transformed with pSM013	this study
SMY068	*MATa/alpha;* YCp*ARS605*	SMY037 transformed with pSM014	this study
T9394	*MATa, DBF4-9myc::hphNT1, RAD5, BUD4, leu2, ura3, trp1, ade2, his3 dbf4-9myc*	*dbf4-9myc* W303 haploid	Tanaka lab
SMY071	*MATa lys2 ura3 ho::LYS2 trp1:: hisG DBF4-9myc::hphNT1 dbf4-9myc*	*dbf4-9myc* SK1 haploid	this study
SMY076	*MATalpha lys2 ura3 ho::LYS2 trp1:: hisG DBF4-9myc::hphNT1 dbf4-9myc*	*dbf4-9myc* SK1 haploid	this study
T9791	*MATa, RAD5, BUD4, leu2, ura3, trp1, ade2, his3 ctf19::KanMX*	*ctf19::KanMX* W303 haploid	Tanaka lab
SMY079	*MATa lys2 ura3 ho:: LYS2 leu2-R his4-X arg4-Bgl ctf19::KanMX*	*ctf19::KanMX* SK1 haploid	this study
SMY080	*MATalpha lys2 ura3 ho::LYS2 trp1:: hisG ctf19::KanMX*	*ctf19::KanMX* SK1 haploid	this study
SMY084	*MATa/alpha lys2/lys2 ura3/ura3 ho::LYS2/ho::LYS2 trp1::hisG/TRP1 leu2-R/LEU2 his4-X/HIS4 arg4-Bgl/ARS4 dbf4-9myc::hphNT1/dbf4-9myc::hphNT1*	*dbf4-9myc* SK1 diploid (SMY071 x SMY074)	this study
SMY086	*MATa/alpha lys2/lys2 ura3/ura3 ho::LYS2/ho::LYS2 trp1::hisG/TRP1 leu2-R/LEU2 his4-X/HIS4 arg4-Bgl/ARS4 ctf19::KanMX/ctf19::KanMX*	*ctf19::KanMX* SK1 diploid (SMY077 x SMY080)	this study
SMY089	*MATa/alpha;* YCp*ARS427.5*	SMY037 transformed with pSM032	this study
SMY097	*MATa/alpha;* YCp*ARS427.5*	SMY037 transformed with pSM046	this study
SMY092	*MATa/alpha;* YCp*ARS427.5*	SMY037 transformed with pSM043	this study
SMY095	*MATa/alpha;* YCp*ARS218*	SMY037 transformed with pSM034	this study
SMY100	*MATa/alpha;* YCp*ARS427.5*	SMY037 transformed with pSM052	this study
SMY104	*MATa/alpha;* YCp*ARS430.5*	SMY037 transformed with pSM061	this study
SMY106	*MATa/alpha;* YCp*ARS509*	SMY037 transformed with pSM063	this study
SMY108	*MATa/alpha;* YCp*ARS605*	SMY037 transformed with pSM066	this study

SMY109	*MATa/alpha;* YCp*ARS218*	SMY037 transformed with pSM067	this study
SMY110	*MATa/alpha;* YCp*ARS227*	SMY037 transformed with pSM070	this study
SMY114	*MATa/alpha;* YCp*ARS430.5*	SMY037 transformed with pSM074	this study
SMY115	*MATa/alpha;* YCp*ARS218*	SMY037 transformed with pSM078	this study
YJL030W	*BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YJL030w::kanMX4*	*mad2Δ::kanMX BY4741 strain*	Euroscarf
SMY117	*MATalpha lys2 ura3 ho::LYS2 trp1:: hisG mad2Δ::kanMX*	*mad2Δ::kanMX* SK1 haploid (*mad2Δ* SMY035)	this study
SMY121	*MATa lys2 ura3 ho::LYS2 trp1:: hisG mad2Δ::kanMX*	*mad2Δ::kanMX* SK1 haploid (*mad2Δ* SMY036)	this study
SMY123	*MATa/alpha lys2/lys2 ura3/ura3 ho::LYS2/ho::LYS2 trp1::hisG/TRP1 leu2-R/LEU2 his4-X/HIS4 arg4-Bgl/ARS4 mad2Δ::kanMX/mad2Δ::kanMX*	*mad2Δ::kanMX* SK1 diploid (SMY117 x SMY119)	this study
SMY131	*MATa/alpha;* YCp*ARS430.*5	SMY037 transformed with pSM081	this study
SMY133	*MATa/alpha;* YCp*ARS416*	SMY037 transformed with pCN9	this study
SMY135	*MATa/alpha;* YCp*ARS416*	SMY037 transformed with pARS1.3	this study
SMY137	*MATa/alpha;* YCp*ARS1021*	SMY037 transformed with pCN10	this study
SMY139	*MATa/alpha;* YCp*ARS1021*	SMY037 transformed with pARS121.1	this study
SMY141	*MATa/alpha lys2/lys2 ura3 ho:: LYS2/ura3 ho:: LYS2 LEU2/leu2-R trp1::hisG/TRP1 HIS4/his4-X ARG4/arg4-Bgl MAD2/mad2Δ::kanMX DBF4/DBF4-9myc::hphNT1*	*mad2Δ* Dbf4 tagged SK1 diploid (SMY121 x SMY076)	this study
SMY143	*MATa/alpha lys2/lys2 ura3 ho:: LYS2/ura3 ho:: LYS2 LEU2/leu2-R trp1::hisG/TRP1 HIS4/his4-X ARG4/arg4-Bgl CTF19/ctf19::KanMX DBF4/DBF4-9myc::hphNT1*	*ctf19Δ* Dbf4 tagged SK1 diploid (SMY079 x SMY076)	this study
SMY147	*MATa lys2 ura3 ho:: LYS2 leu2-R his4-X arg4-Bgl mad2Δ::kanMX DBF4-9myc::hphNT1*	*mad2Δ* Dbf4 tagged SK1 haploid (spore dissected from SMY141, SMY148 is also from the same tetrad as SMY147)	this study
SMY148	*MATa lys2 ura3 ho:: LYS2 leu2-R his4-X arg4-Bgl mad2Δ::kanMX DBF4-9myc::hphNT1*	*mad2Δ* dbf4 tagged SK1 haploid (spore dissected from SMY141, SMY148 is also from the same tetrad as SMY147)	this study
SMY155	*MATa lys2 ura3 ho:: LYS2 leu2-R his4-X arg4-Bgl ctf19::KanMX DBF4-9myc::hphNT1*	*ctf19Δ* dbf4 tagged SK1 haploid (spore dissected from SMY143, SMY156 is also from the same tetrad as SMY155)	this study
SMY156	*MATalpha lys2 ura3 ho::LYS2 trp1:: hisG DBF4-9myc::hphNT1 ctf19::KanMX*	*ctf19Δ* dbf4 tagged SK1 haploid (spore dissected from SMY143, SMY156 is also from the same tetrad as SMY155)	this study
SMY160	*lys2/lys2 ura3 ho:: LYS2/ura3 ho:: LYS2 LEU2/leu2-R trp1::hisG/TRP1 HIS4/his4-X ARG4/arg4-Bgl mad2Δ::kanMX/mad2Δ::kanMX DBF4-9myc::hphNT1/DBF4-9myc::hphNT1*	*mad2Δ* dbf4 tagged SK1 diploid (SMY147 x SMY148)	this study
SMY162	*lys2/lys2 ura3 ho:: LYS2/ura3 ho:: LYS2 LEU2/leu2-R trp1::hisG/TRP1 HIS4/his4-X ARG4/arg4-Bgl ctf19::KanMX/ctf19::KanMX DBF4-9myc::hphNT1/DBF4-9myc::hphNT1*	*ctf19Δ* dbf4 tagged SK1 diploid (SMY155 x SMY156)	this study
SMY158	*MATa/alpha; YCp*ARS227	SMY037 transformed with pSM083	this study

Table 4: Plasmids used in this study

Plasmid	Description	Source
pMSH001	*ARS605* in YCp-lacZ	CAN Lab
pMSH006	*ARS605* in YCp-lacZ	CAN Lab
pMSH007	*ARS607* in YCp-lacZ	CAN Lab
pCA011	*ARS607* cut out of CA001 with *Not*I and *Spe*I, cloned into YCp-lacZ	CAN Lab
pSM008	SGRP clone SK1-65b03 amplified with SM001 & SM002, cloned into pGEM-T easy vector	this study
pSM005	*ARS605* cut out of pSM008 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM006	*ARS605* cut out of pSM008 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM011	SGRP clone SK1-85a08 amplified with SM001 & SM002, cloned into pGEM-T easy vector	this study

pSM013	*ARS605* cut out of pSM011 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM012	SGRP clone SK1-85a08 amplified with SM001 & SM002, cloned into pGEM-T easy vector	this study
pSM014	*ARS605* cut out of pSM012 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM015	SGRP clone SK1-16e18 amplified with SM003 & SM004, cloned into pGEM-T easy vector	
pSM032	*ARS427.5* cut out of pSM015 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM022	SGRP clone SK1-16e18 amplified with SM005 & SM006, cloned into pGEM-T easy vector	this study
pSM046	*ARS427.5* cut out of pSM022 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM034	SK1 strain genomic DNA is amplified with SM007 & SM008, cloned into pGEM-T easy vector	this study
pSM045	*ARS218* cut out of pSM034 with *NotI* and *SpeI,* cloned into YCp-lacZ.	this study
pSM048	SGRP clone SK1-16e18 amplified with SM003 & SM005, cloned into pGEM-T easy vector	this study
pSM052	*ARS427.5* cut out of pSM048 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM056	SK1 strain genomic DNA is amplified with SM024 & SM025, cloned into pGEM-T easy vector	this study
pSM062	*ARS430.5* cut out of pSM056 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM059	SGRP clone SK1-83h21 amplified with CA180 & CA181, cloned into pGEM-T easy vector	this study
pSM074	*ARS430.5* cut out of pSM059 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM057	SK1 strain genomic DNA is amplified with SM030 and SM031, cloned into pGEM-T easy vector	this study
pSM063	*proARS509* cut out of pSM057 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM058	SGRP clone SK1-65b03 amplified with SM036 & SM037, cloned into pGEM-T easy vector	this study
pSM066	*ARS605* cut out of pSM058 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM060	SK1 strain genomic DNA is amplified with SM038 and SM039, cloned into pGEM-T easy vector	this study
pSM067	*ARS218* cut out of pSM060 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM069	SK1 strain genomic DNA is amplified with SM026 and SM027, cloned into pGEM-T easy vector	this study
pSM070	*ARS227* cut out of pSM069 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM071	SK1 strain genomic DNA is amplified with SM040 and SM041, cloned into pGEM-T easy vector	this study
pSM078	*ARS218* cut out of pSM071 with *Not*I and *Spe*I, cloned into YCp-lacZ	this study
pSM081	SGRP clone SK1-21n19, spanning *ZIP1* gene for *ARS*430.5 is cloned into YCp-lacZ	this study
pSM083	SGRP clone SK1-70j10, spanning *ZIP1* gene for *ARS*227 is cloned into YCp-lacZ	this study
pCN9	*ARS*416 smaller insert cloned into YIp-5'5	CAN Lab
pARS1.3	*ARS*416 larger insert cloned into YIp-5'5	Donaldson Lab
pCN10	*ARS*1021 smaller insert cloned into YIp-5'5	CAN Lab
pARS121.1	*ARS*1021 larger insert cloned into YIp-5'5	Donaldson Lab
YCp-lacZ	plasmid backbone used for ARS assays; lacks yeast replication origin	CAN Lab
pGEM T easy	Vector routinely used for cloning of PCR products	Promega
pEH161	Plasmid used to express *HO* to generate homozygous diploid	Eva Hoffmann's Lab

Table 5: Oligonucleotides used in this study

Oligo	Description	Sequence
SM001	5'*ARS605* small fragment	GATGCCCAGACACAGTCTTC
SM002	3'*ARS605* small fragment	GGCCCTAGAAGAATTGAAAGC
SM003	5' *ARS427.5* small fragment	TTCCTTTGATAGTCTTCAATAATTCTG
SM004	3' *ARS427.5* small fragment	ACAATTCACAAAGCTCTGTTGT
SM005	5' *ARS427.5* large fragment	ATAAGACAATTTGTGGTTGCCTTTA
SM006	3' *ARS427.5* large fragment	TCTCTATCTTGCTCACTTCACATTG
SM007	5' *ARS218* small fragment	CACTTGGAAAAGAGAATATTTGGC
SM008	3' *ARS218* small fragment	GGCTCGAGCTAGTAGCATTTTCAGTAAACGAA
SM009	5' *ARS218* large fragment	TATGGAGGAAACAAAATTACTAGCG
SM010	3' *ARS218* large fragment	GGCTCGAGCTGAAGAAGACATGATAGCAGTTGA

SM011	5' *DBF4*	TGCTTTTGGTTCATATTTAGAAAAGA
SM012	3' *DBF4*	TATCACTAAAAGCTACTGCACTTTACGTC
SM013	5' *CTF19*	GTCGGCAAAGAACGCAAA
SM014	3' *CTF19*	AGTTGGCAATGGCAAATGGA
SM015	'A' primer for 5' *CTF19*	AAAGAAAAGCTGTACAGGGAAAAAT
SM016	'D' primer for 3' *CTF19*	TTACCACGGGAAGAGAATACTACAG
SM017	Forward primer for 'A' primer of *CTF19*	TGCGACTGCACTGTGTTGTA
SM018	Reverse primer for 'D' primer of *CTF19*	AAGGCAGGGTTTTGTTCTCA
SM019	3' *DBF4*	GAGACGGGCAAAGATAGTGC
CN224	*DBF4* primer – 3' of ORF	GTGGGTACTCGATCATTATTTGTTC
SM020	5' for primer CN224 of *DBF4*	AAAACTGGGACATTAACCGC
SM021	3' for primer SM019 of *DBF4*	AAAGACGAAGCCCCTTCGAAA
SM022	5' *ARS430.5*	AATGGGATTCAAGCCTCGAC
SM023	3' *ARS430.5*	ATTTCCTTCTCCTTTTCTTGCTT
SM024	5' ARS430.5	CGACTACAGTCGTATGCTTCCA
SM025	3' ARS430.5	TGTTGTGATGTTCAAATTGTTGAG
SM026	5' ARS227	TTCTCCTTTAAAAAGCAAGAGC
SM027	3' ARS227	TTTCTGACGATAAGAAAGGTGA
SM028	5' ARS227	GCGACTCATAATTGCGTGAA
SM029	3' ARS227	TGGTTTTGTTCCCATCCATT
SM030	5' pro*ARS509*	GTTCCAATACCCACCACACC
SM031	3' pro*ARS509*	ACCAACCTGGAATTGGTTCA
SM032	5' pro*ARS509*	CCTCGTCCTGCCTTATCCTT
SM033	3' pro*ARS509*	TTATGGTAATGGCCCTCCTG
SM034	5'*ARS605*	TCGGTATTGTGTAATTATTTTCTTTCA
SM035	3'*ARS605*	TTGCTCTTTGTGCTGCATCT
SM036	5' *ARS605*	TCGCTAATTAAACGGCTTTGA
SM037	3' *ARS605*	CTGTAGCTGATCAACGCAAA
SM038	5' *ARS218*	TTGGAAATGAACAAATTGAAAAA
SM039	3' *ARS218*	GCATTTTCAGTAAACGAAATTGTTA
SM040	5' *ARS218*	GCACCGGTGTATTAACATATATAAGGA
SM041	3' *ARS218*	GCATTTTCAGTAAACGAAATTGTTA
SM042	5'*MAD2*	TTTCGTCTTGAACTCTCTTTTGTCT
SM043	3'*MAD2*	ATCTTTTCATATTCAAGTGGGACAA
SM044	5' *MAD2* large	CAACTCAGAAGCCAAACTCG
SM045	3' *MAD2* large	CTCTGGATTCCGCTGATGA
SM046	5' *MAD2* outside SM042 for confirming mad2 deletion	TCACGACAGCAAGTTCAGTT
SM047	3' *MAD2* outside SM045 for confirming mad2 deletion	GGATGCAAGATCCGAATTGGTC
SM048	3' *ZIP1* alternate to SM023	TGTATTCGCACAAAACGATTTCA
SM049	5' *ARS605* alternate to SM034	TGTCTTGAATCTCGAATTAGTAGATTG
SM050	3' start of *SPO22* gene	AAGGCTATGTTCTAAAGTGATCAACA
SM051	5' nimARS region of *SPO75* gene	GTCTGTGCCCGACGATAAAT
SM052	3' nimARS region of *SPO75* gene	CGCAAAGACATGCTGGAAT
SM053	5' nimARS region of *SPO75* gene, similar to SM051 but specific for SK1	GTCTGTGCCCGACGATTTAT

2.4 Molecular genetic methods

2.4.1 Selection and recovery of SGRP clones

The plasmids are from whole genome short-gun libraries, size selected to have an average insert of 4 kb. All the plasmids were sequenced by the Sanger Center (Cambridge, UK) with both a forward and reverse read. Plasmid libraries are available from 37 different *S.cerevisiae* strains; here I have used plasmids generated from the strain SK1. The plasmids were obtained from SGRP (The Saccharomyces Genome Resequencing Project) {Liti, 2009

#1}. 0.5 µl of the required plasmid was recovered by transformation using a *E.coli* by heat shock protocol (see 2.4.2).

2.4.2 Heat Shock Transformation of *E. coli* cells

Recovered plasmids, ligations and other plasmid DNA were transformed into chemical competent *E.coli* cells (XL-1 Blue cells from Stratagene) using a heat shock transformation protocol. In short, *E.coli* cells were thawed on ice and aliquots were taken as required. Approximately 0.5-1.0 µl of the sample was added to the cells as required and incubated on ice for 20-30 minutes to make the plasmid adhere to the cell wall. The mix was then heat shocked at 42ºC for a minute and then immediately put on ice for 5 minutes. Heat shock allows the cell pores to open and allow the entry of plasmid into the cells. Heat-pulse step had two important roles on DNA entry: (a) Release of lipids and consequent formation of pores on cell surface, which helped DNA to cross outer membrane barrier, and (B) lowering of membrane potential, which facilitated DNA to cross inner membrane of *E. coli* (Panja et al., 2008). Further incubation on ice after the heat shock, allows the closure of pores preventing plasmid escape. 1ml of mu media was then added to the cells and incubated at 37ºC for an hour. Cells were then pelleted and plated on selection media (e.g. LB Amp) and incubated overnight at 37ºC.

2.4.3 Preparation of electrically competent cells

Sterile water, 50ml falcon tubes and 10% glycerol were all pre-chilled the previous day. On the day of preparation, the centrifuge and the rotor were pre-cooled to 4ºC. A 10ml culture of *E.coli* (XL1-Blue) cells was inoculated and grown overnight at 37ºC with shaking. On the morning of the preparation, the culture was diluted 1 in 100 in 200ml of LB broth and continued to grow at 37ºC. The culture was cooled on ice once it reached an OD_{600} of 0.5-0.7. Cool conditions were from then on maintained through out the preparation. The culture was divided into 50ml falcons and cells were then harvested on a table top centrifuge at 3500 rpm for 5 minutes, at 4ºC. The supernatant was discarded and the cells were resuspended in a total of 200 ml of cold sterile water. Cells were harvested at the same conditions and the washing step was repeated in a total of 100ml of cold sterile water. The suspension was harvested and the cell pellet was now pooled in 4ml of cold 10% glycerol. The suspension was then centrifuged and resuspended again in 500 µl of cold 10% glycerol. Cells were aliquoted into 50 µl lots on ice and frozen at -80ºC for use as required.

2.4.4 Electrical transformation of *E.coli* cells (Electroporation)

Some of the recovered plasmids were recovered into bacterial cells by electrical transformation. For the electroporation/electro transformation, electrically competent *E.coli* cells were used. The cells were gently thawed at room temperature and placed on ice. Bio-Rad Micropulse machine was used for electroporation. Program of the machine was set to EC2 in the bacterial settings menu (V = 1.8kV). *E.coli* cells were added to the DNA, mixed well and left on ice for 30 seconds. The cuvette was dried and the content was transferred into the cuvette and mixed. Cuvette was placed in the safety chamber and pulsed once until a beep sound was heard. The 'time constant' after the pulsing should be between 4 and 5 seconds. Immediately 1ml of SOC (or LB) media was added to the cuvette, contents were mixed and returned to the culture tube. The tubes were allowed to shake gently at ~225rpm at 37°C for 1 hour. Cells were mixed in a microfuge at 8000 rpm for 1 minute and plated on selective medium plates and incubated at 37°C overnight.

2.4.5 Plasmid DNA extraction from *E.coli* cells

Nucleospin plasmid DNA extraction (Machery-Nagel NucleoSpin® Plasmid Kit) protocol was used for extracting the DNA from *E.coli* and is described below:

Cells were grown overnight in 5 ml liquid YPD culture at 37°C. Cells were harvested by centrifugation at 4600 rpm for 5 minutes at room temperature. Supernatant was removed completely with a micropipette. The cell pellet was then resuspended in 250 µl of resuspension buffer, A1. The cell pellet was mixed completely by vortexing so that there were no cell clumps before adding the lysis buffer. Then 250 µl of lysis buffer, A2 was added, mixed gently and incubated at room temperature for 5 minutes. Then 300 µl of neutralization buffer, A3 was added and mixed gently by inverting up and down. The mixture was centrifuged for 10 minutes at 11,000 rpm. The nucleospin plasmid column was placed in the collection tube and a maximum of 750 µl of the supernatant was transferred on to the column and centrifuged for 1 minute at 11,000 rpm. The flow through was discarded and 600 µl of wash buffer; A4 was added to wash the silica membrane. After centrifugation for 1 minute at 11,000 rpm, the flow through was discarded and the colum was centrifuged again for 2 minutes at 11,000 rpm to dry the silica membrane. The nucleospin plasmid column was now placed back into a fresh 1.5 ml tube. DNA was eluted by adding 50 µl of elution buffer, AE to the column. This was incubated at room temperature for 1 minute and centrifuged for 1

minute at 11,000 rpm. The eluted DNA was stored at -20°C or used immediately. The concentration of the DNA was determined by testing with a Nanodrop Spectrophotometer.

2.4.6 Restriction Digestion of DNA

Restriction digestion of DNA was carried out according to the manufacturer's recommendations (NEB). All the digests were carried out in a total volume of 10 µl - 40 µl, containing 2-10 units of enzyme per 10 µl reaction. All digests were carried out for 1 – 2 hours at 37ºC, unless different temperature was optimized for the enzyme.

2.4.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used routinely to separate DNA molecules based on size. 0.8% agarose gels were prepared with TBE buffer (10× buffer: 108 g Tris, 55 g boric acid, 40 ml EDTA 0.5 M (pH 8.0), made up with 1000 ml of water). Agarose gels contained 0.3 µg/ml ethidium bromide to allow visualization of DNA fragments using a Bio-Rad Gel Doc. DNA samples were supplemented with 1× loading dye (5× dye: 1 g Ficoll type 400, 20 ml EDTA 0.5 M, 100 mg bromophenol blue, 100 mg xylene cyanol, 5 ml 10% SDS, water added to 100 ml). Alongside the samples, 0.5 µg of a DNA ladder (Bioline, Hyperladder I – V depending on the expected size of the product) was loaded on to the gel as a control for sizes. Electrophoresis was performed in 1× TBE buffer at 75 – 115 V until DNA fragment separation was sufficient.

2.4.8 DNA extraction from agarose gels

If required, following electrophoresis, the DNA fragments were excised from the gel using a scalpel. QIAGEN gel extraction kit was used to extract the DNA from the gel following the manufacturer's instructions. The excised gel slice was melted at 50ºC in the binding solution provided in the kit, which allows binding of the DNA to the silica membrane of the column. The bound DNA was then washed with the wash buffer supplemented with ethanol and eluted in 35 µl or 50 µl of elution buffer depending on the concentration of the product required.

2.4.9 Ethanol precipitation of DNA

Ethanol precipitation of DNA allows the purification and concentration of the sample from a dilute solution by removing the unwanted salts. 0.3M concentrations of sodium acetate and 3X volumes of 100% ethanol were added to the DNA sample and supernatant was discarded after centrifugation. The resultant pellet was washed with 70% ethanol, centrifuged and the supernatant was discarded. The pellet was air dried and resuspended in an appropriate volume of TE buffer (10mM Tris-HCL (pH 8.0), 1mM EDTA).

2.4.10 DNA ligation

Ligations were performed to clone specific sequences (PCR products) into desired vectors like pGEM®-T easy or YCp-lacZ. The pGEM®-T Easy Vector System (Promega) supplies linearized pGEM®-T vector featuring a single 3'-terminal thymidine overhang at both ends. These T-overhangs are compatible to the 5'-terminal adenine of PCR products amplified with *Taq* polymerase, thus allowing high efficiency ligations. Ligations were performed according to the manufacturer's instruction, but the reaction volumes were halved. Ligation of a DNA fragment into YCp-lacZ was carried out using T4 DNA ligase (NEB) according to the manufacturer's protocol. Prior to the ligation, digests of insert and vector with the restriction enzyme(s) were performed to created compatible overhangs. Ligation reactions were incubated for 2 hours at room temperature or at 15 °C overnight. Ligation products were recovered by transformation into *E.coli*.

2.4.11 Concentration of ligated samples by Drop Dialysis

The ligated samples were concentrated in some cases prior to electroporation, to remove the excess salts present by drop dialysis. Millipore® VMWP02500 MF-Millipore™ DNA Filters were used. A beaker with sterile distilled water was used, and the membrane filters were floated on the water with the shiny side up and allowed to soak for 5 minutes. 50% of the ligation sample was added to the membrane as a drop and left for 30 minutes for dialysis to occur. The sample was then carefully retrieved.

2.4.12 Polymerase Chain Reaction (PCR)

PCRs were performed either to amplify specific DNA fragments or to genotype yeast strains (section 2.5.4). BIOTAQ™ DNA polymerase (Bioline) was mostly used, however Expand High Fidelity PCR System (Roche) and Q5® High-Fidelity DNA Polymerase (NEB) were used for error free products. A standard PCR reaction was carried out in a 10-50 µl reaction volume consisting of 10-200 ng of template DNA, dNTPs (dATP, dCTP, dTTP, dGTP) at a final concentration of 0.25 mM each, 1 µM each of two oligonucleotide primers (forward and reverse primers), 1 unit of Taq polymerase in 1X reaction buffer (supplemented with $MgCl_2$ supplied by Bioline) made to final volume with sterile distilled water. A typical PCR program consists of an initial denaturing step at 94 °C for 4 minutes followed by thirty cycles of DNA denaturation at 94 °C for 30 seconds, primer annealing at 53 °C for 30 seconds and primer elongation at 72 °C for 1 minute per amplified 1 kb of DNA. All PCR reactions were run on a Bio Rad DNA Engine Thermal Cycler.

2.4.13 Fusion PCR

The technique of fusion PCR was used to swap the promoters of two genes. Complementary primers were designed using the www.primer3plus.com website. The forward and reverse primers were designed in such a way that they contain the restriction sites for further subcloning into the empty vector. Roche Expand High Fidelity PCR system was used to avoid error in PCR products.

The parameters used for fusion PCR are:

Step	Teperature (ºC)	Time	Cycles
1	95	4 min	1
2	95	30 sec	1
3	52	30 sec	1
4	72	90 sec	1
5	Cycle to step 2	-	4
6	95	30 sec	1
7	56 – 60 gradient	30 sec	1
8	72	90 sec	1
9	Cycle to step 6	-	24
10	72	10 min	1
11	15	forever	-

2.4.14 PCR clean up

A clean up procedure was carried out after the PCR, when the sample was required free from excess primers, DNA polymerase, nucleotides etc., which might interfere with the later manipulations. GenElute™ PCR Clean-Up Kit was used for the clean up process, where the DNA was made to bind the silica membrane in the spin column using the binding solution. The DNA was then washed with wash solution and eluted in 35-50 µl of elution solution depending on the concentration required.

2.4.15 DNA Quantification

The plasmid DNA and PCR products were quantified on a spectrophotometer (Thermo Scientific NanoDrop™ 1000 Spectrophotometer) or by agarose gel electrophoresis.

2.4.16 DNA Sequencing

When required the DNA sequence of plasmids and amplified PCR products were verified by Sanger sequencing. All the sequencing reactions were performed by Source BioScience (Nottingham branch). High throughput (drop) sequencing was used to generate genome wide replication profiles. Deep sequencing was performed by High Throughput Genomics group (University of Oxford) on an Illumina HiSeq.

2.5 Yeast Methods

2.5.1 Growth conditions

Yeast strains were recovered in patches or streaked to single colonies on YPD or selective media plates. Plates were incubated at 30ºC for 2-3 days and could then be stored at 4ºC for up to two weeks. Strains were stored by picking up a single colony and resuspending it in 1 ml of 25% glycerol and frozen at -80ºC for long-term storage. Most yeast methods require liquid cultures. 5 ml cultures were set up either in YPD or selective media (ura drop out media usually) using cells from a single colony and grown in a shaking air incubator overnight (200 rpm), which was used as a starter culture for setting up cultures on the day of the experiment. Cultures of desired cell density and volume (25

ml – 500 ml) were set up and grown in shaking water baths. Doubling times were calculated based on OD_{600} readings on a BioMate™ 3S Spectrophotometer, taken during exponential growth phase.

2.5.2 High efficiency transformation of yeast cells

Yeast cells were transformed by high efficiency Lithium Acetate transformation using the protocol from Daniel Geitz (Geitz, 2007). Yeast cells were transformed with plasmids to test ARS activity or with a linear DNA for targeted integration in to the genome by homologous recombination. A 5 ml starter culture was set up in YPD liquid media and was incubated overnight at 30°C in a rotary shaker at 200 rpm. After overnight growth of nearly 12-16 hours, the optical density of the cells was checked in a spectrophotometer. The cells were ready for transformation at an OD_{600} of 0.5-1.0. Once the cells reached the required OD_{600}, they were harvested by centrifugation at 3000 rpm for 5 minutes at 20°C to pellet the cells. Supernatant was discarded and the pellet was resuspended in 50 ml of sterile water and centrifuged at 3000 rpm for 5 minutes at 20°C. The supernatant was discarded, washing and centrifugation steps were repeated as above. The cells were then resuspended in 2 ml of sterile water and the cell suspension was transferred to 2 ml tube and centrifuged for 1 minute at 13,000 rpm, supernatant was discarded and the cells were resuspended in 2 ml of sterile water. 100 µl aliquots of cell suspension were taken into each 1.5 ml tube; one for each transformation, centrifuged for 1 minute at 13,000 rpm and the supernatant was discarded. Single stranded DNA was denatured in a boiling water bath for 5 minutes and was chilled immediately on an ice bath.

Transformation mix was prepared according to the following recipe.

Transformation mix components	Volume (µl)
50% w/v PEG	240
1.0M LiAc	36
Single stranded carrier DNA	50
Plasmid DNA plus sterile water	34
Total Volume	360

Plasmid DNA plus sterile water was added separately for multiple plasmids. In such cases, 326 µl of transformation mix and 34 µl of plasmid DNA plus sterile water were added separately to the cell pellet and vortexed vigorously. DNA concentration of 200ng/µl was used. The tubes were placed in a water bath at 42°C and incubated for 40 minutes. The tubes were then centrifuges at 13,000 rpm for 1 minute. Supernatant was discarded and the cells were suspended in 200 µl of sterile water and vortexed vigorously. The cell suspension was plated on appropriate selective media, spread evenly using glass beads. The plates were incubated at 30°C for 3-4 days, until colonies were clearly visible.

2.5.3 Quick transformation of yeast cells

A quick transformation of yeast cells was performed to obtain yeast strains by mating type switching through transient expression of the *HO* gene. The quick transformation procedure was used when only a few transformants were required. (Gietz and Schiestl, 2007). The difference of the protocol compared to the standard transformation procedure was that the cells were taken directly from a plate rather than growing them in a liquid culture and washing them further. A fresh patch of yeast cells were grown at 30ºC on agar plates for 2-3 days. Cells were scraped directly from the plate and resuspended in 1ml of sterile water, such that they contain approximately 5 x 10^8 cells. Cells were then pelleted at 13,000g for 30 sec and supernatant was discarded. The transformation mix was prepared as indicated in the high efficiency transformation protocol (2.5.2), the pellet was resuspended in transformation mix and DNA + water were added to it and heat shocked at 42ºC for 20 – 180 minutes. Cells were the centrifuged, supernatant discarded and pellet was suspended in sterile water and plated on appropriate selection plates.

2.5.4 Colony PCR

Yeast strains were genotyped quickly, by colony PCR as described previously (Stansfield and Stark, 2007). A small number of yeast cells from a single colony was resuspended in 10 µl of SPZ buffer in a 200 ml tube. The suspension was exposed to a three-step program in a PCR cycler – 30 minutes at 37ºC to spheroplast the cells, 5 minutes at 95ºC to degrade proteins and 15ºC ‘forever’ to preserve the sample. 95 µl of water were added to the suspension. The sample was frozen or 1 µl was used directly as template in a PCR.

2.5.5 Genomic DNA extraction from yeast

Yeast genomic DNA was extracted for use as a template for PCR's. A 1.5 ml yeast culture was grown overnight in a sterile eppendorf tube. The cells were pelleted by centrifugation for 30 seconds at 13,000 rpm. Supernatant was discarded and the cells were suspended in 500 mM Sorbitol in TE buffer. The cells were treated with Ribonuclease A and Zymolase at 37ºC for 1 hr to lyse the cells and digest RNA, incubated at 37ºC for 1 hr. The cells were then washed with SDS and incubated for 20 minutes at 65ºC, to remove membrane lipids. The cells were further treated with 5M potassium acetate and centrifuged for 10 minutes at 13000 rpm. To the resultant supernatant, propanol was added and incubated on ice for 10 minutes to precipitate DNA. The whole mix was then centrifuged and the pellet washed with 70% ethanol. The sample was centrifuged and the pellet was allowed to air dry, to remove residual ethanol. The genomic DNA was resuspended in 100 µl of TE buffer and incubated at 65ºC for 10 minutes to allow the pellet to dissolve.

2.5.6 ARS assay

The existence of a functional replication origin can be determined by ARS (Autonomously Replicating Sequence) assays. ARS assay is a conventional and recombination based assay, to determine the presence of ARS in a genome sequence, as described in published protocols (Stinchcomb et al., 1979, Nieduszynski and Donaldson, 2009). The fragments of interest were amplified by PCR and cloned into pGEM®-T. The clone was recovered in *E.coli* and confirmed by Sanger Sequencing. For the conventional ARS assays, the desired insert was excised from the plasmid using appropriate restriction enzymes and purified using a column (QIAGEN PCR Purification Kit). A ligation reaction between the linearized vector (YCp-lacZ) and ARS insert, with a molar ratio of 1:3 was set up and this ligation mix was used to transform *E.coli*. The plasmid was extracted, sequence verified and transformed into yeast. For the recombination-based ARS assay, uncut pGEM®-T clones (10 – 34 µl of purified plasmids) together with linearized YCp-lacZ (100 ng for *S. cerevisiae*) were used to co-transform yeast cells (strain : BY4741). Transformations were plated on uracil lacking media. The transformed yeast strains are auxotrophic for the Orotidine-5'-phosphate (OMP) decarboxylase (encoded by *URA3*) and thus colony growth depends on the functional copy of *URA3* on YCp-lacZ. However, YCp-lacZ does not contain a yeast replication origin and it can only be maintained if a functional ARS is present in the tested insert as a result of 'gap repair' (see 2.5.7).

2.5.7 Gap Repair

Cloning by gap-repair is an effective way of constructing plasmids in budding yeast. Generally, restriction enzymes and DNA ligase are used for cloning a specific gene into a plasmid. To clone specific yeast genomic fragments, these are usually amplified by polymerase chain reaction (PCR), using primers that incorporate unique restriction sites. The amplified fragments are then treated with the respective restriction enzymes and ligated to similarly digested vectors. While practical and efficient, this procedure can incorporate base substitutions in the amplified fragments due to the intrinsic error rates of the DNA polymerases used in PCR. Another disadvantage is that the length of the fragment to be cloned is limited by the efficacy of the DNA polymerase used (normally up to ~5 kb, and ~20 kb with specialized mixes). Gap Repair has been used as an alternative to molecular cloning techniques for the construction of specific plasmids. In the budding yeast *Saccharomyces cerevisiae*, cloning by gap repair is widely used in plasmid construction (Figure 13). Gap repair depends on the ability of the yeast to repair gapped DNA sequences *in vivo* by homologous recombination. By this method, DNA fragments with flanking homology to the target plasmid can be cloned directly into a linearized plasmid by homologous recombination without the need for *in vitro* DNA ligation. Gap repair cloning is more simple and convenient because short (20bp) tracts of flanking homology are sufficient for effective gap-repair cloning in budding yeast. This method is particularly useful in cases where suitable restriction sites are not available. However, in contrast to the budding yeast, gap repair cloning is not widely used in the fission yeast *S pombe*, which is due to the requirement of longer region of flanking homology.

Gap-repair, gap-filling or *in vivo* cloning allows the error-free construction of plasmids in yeast cells, which are able to restore gapped plasmids using homologous sequences as templates. This procedure typically starts with construction of a targeting plasmid into which the inserts (cognate sequence) will be cloned and thus harbor the respective flanking homologous sequences. The targeting plasmid is linearized via digestion with appropriate restriction enzymes to generate the free ends that will target the cognate sequence and promote gap repair. This method has proved efficient and accurate and has been used to clone genomic fragments, and restriction- or PCR-derived fragments that are introduced with the gapped plasmid by co- transformation. To increase the accuracy of gap repair induction, it is crucial to keep the total amount of DNA to less than 200ng, especially when co-transforming yeast with several fragments. Therefore, to obtain a high number of transformants, it is preferable to perform several independent transformations.

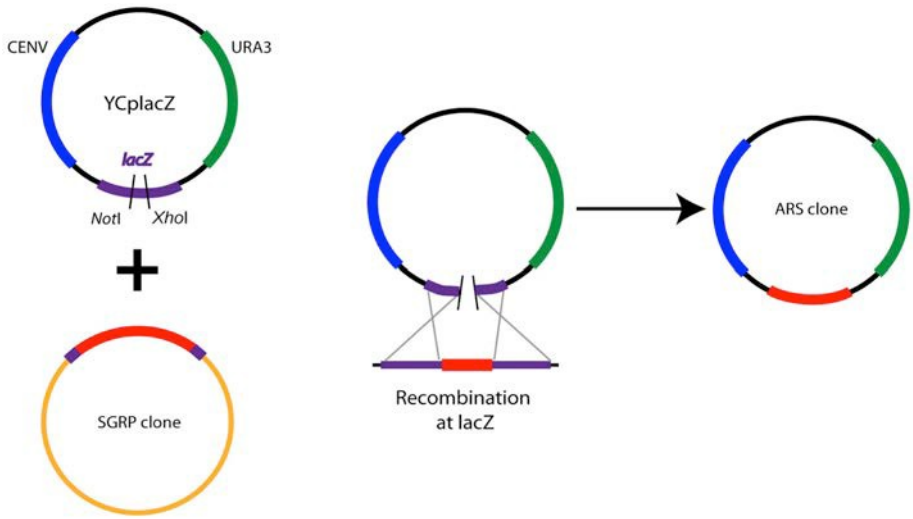


Figure 13: Schematic representation of Gap Repair. YCplacZ is an ARS assay vector, which has a centromere and a selectable marker but lacks ARS activity. The gapped vector is repaired with SGRP clone carrying *ARS* activity.

The assay vector YCplacZ was cut with enzymes *Not I* and *Xho I* by Linearized ARS assay. The vector digestion was confirmed by running on agarose gel. The cut assay vector and the repair vector (SGRP plasmids) were transformed into yeast strain (BY4741) by the yeast transformation protocol and plated on to selective medium (ura-). The transformed single colonies were transferred into –ura liquid medium and incubated at 37°C for plasmid rescue. The extracted plasmid was transformed into the electrically competent *E.coli* cells by electroporation protocol. The DNA was extracted from the transformants by Nucleospin plasmid extraction protocol and the evidence of gap repair was seen by digesting the DNA with specific restriction enzyme *EcoR I.*

Gap-repair ARS Assay

dd H2O	5.8 µl
NEB4	2.0 µl
100X BSA	0.2 µl
YCplacZ (250ng/ µl)	10 µl
Not I	1.0 µl
Xho I	1.0 µl
Total	20 µl

2.5.8 Plasmid Rescue from Saccharomyces cerevisiae

Cells were grown overnight under selective conditions and harvested at 5000 rpm for 5 minutes. Supernatant was discarded and the pellet was resuspended in 100 µl of STET buffer and transferred in to a screw capped microfuge tube. 0.2 g of 0.45 mm glass beads were added to the microfuge tube and vortexed vigorously for 10 minutes. Another 100 µl of STET buffer was added, vortexed and placed in a boiling water bath for 3 mins. Then, it was cooled on ice and centrifuged in a microfuge at 13,000 rpm for 10 mins in cold room. 100 µl of the supernatant was transferred to a fresh tube containing 50 µl of 7.5 M Ammonium Acetate and incubated at -20°C for 1 hour and centrifuged. 100 µl of this supernatant was added to 200 µl of ice cold 100% ethanol, mixed and DNA was recovered by centrifugation. The pellet was washed with 100 µl of 70% ethanol and the pellet was dried and resuspended in 20 µl of sterile water.

2.5.9 Mating type assay

To assess the mating type, the cells were crossed with mating testers of known mating type (a and alpha) **(***ho Mat a / α ura1-1 tyr1-1***)** and genotype, on YPD plates. The plates were incubated overnight at 30°C and were replica plated on to minimal media. Yeast can grow on the minimal media only if they can mate with either of the tester strain. The mating type of the cells can be determined, since mating occurs only between the cells of opposite mating type, dependent on which tester strains can complement it's genotype.

2.5.10 Sporulation and Tetrad Dissection

The yeast cells grown on YPD media, were replica plated on to sporulation media (16 g Potassium Acetate, 1.76 g Yeast extract, 0.4 g Dextrose – D-Glucose) and incubated for 3-4 days at 30°C. Tetrads were dissected by first digesting with 5 µl of zymolase in 95 µl of dissection buffer, incubated at 37°C for 10 minutes (Sherman, 2002). The cells were spread as a thin line on the dissection plates. Separation of the haploid spores was performed under a dissection microscope (SporePlay©, Singer) using a micromanipulator (a glass needle). Spores were allowed to grow for 2 days at 30°C.

2.5.11 Measuring DNA content by flow cytometry

Flow cytometry was used to measure the DNA content, to assess cell ploidy and cell cycle phases of cultures. Cultures with an optical density A_{600} of 0.7, containing 1 ml of cells were pelleted (8000rpm), washed with water and fixed in 70% ethanol. The fixed samples can be stored at 4ºC for at least 2 hours. Cells were pelleted, washed in filter-sterilized 50 mM Sodium Citrate (pH 7.4) and suspended in 500 µl of 50 mM Sodium Citrate. Cell aggregates were separated by sonicating the samples for 3 minutes (intervals of 5 seconds on and 2 seconds off using a MISONIX XL2020 sonicator). Samples were made up to a ml with 50 mM Sodium Citrate and treated with RNase A (0.25 mg/ml from 10 mg/ml stock) and then proteinase K (50 µl of freshly prepared 20 mg/ml stock) for 1 hour at 50ºC. Cells were pelleted (8000 rpm, 1 minute, RT) and resuspended in 1 ml 50 mM sodium citrate containing 1 SYTOX® green nucleic acid stain (Invitrogen). Samples were stored at 4ºC until analyzed using either an Apogee A40 or Coulter FC 500 flow cytometer. Both machines use an argon ion laser (488 nm) with a suitable excitation range for SYTOX® green. The parameters for forward (FS) and side scatter (SS) data acquisition were the same for all samples (FS 896 V, SS 75 V). However, the voltage and thus sensitivity of the fluorescence detector FL1 (emission filter 500 – 556 nm) was optimized for each analyzed strain background (generally 415 V for diploids, 465 – 475 V for haploids).

2.5.12 DNA extraction of cells obtained from FACS

For FACS, cells were subjected to 70% ethanol, RNase A, proteinase K and 10 SYTOX® green nucleic acid stain. Sorted cell samples, obtained from FACS, usually contain ~1 million cells per 1 ml of saline sheath fluid (Beckman Coulter). A 1/3 volume of 100% ethanol was added to aid pelleting of the cells. Samples were aliquoted into microcentrifuge tubes and spun at 13000 rpm for 10 minutes. Cell pellets were resuspended and pooled in a total volume of 500 µl of 50 mM Tris-HCl and 0.1 mM EDTA. Samples were treated with 50 µl of Zymolyase (10 mg/ml) and 5 µl β-mercaptoethanol for 30 minutes at 37ºC to spheroblast the cells. 17 µl of 3M sodium chloride, 12.5 µl of 20% SDS, 10 µl proteinase K (freshly prepared stock of 20 mg/ml in TE) and 5 µl RNase A (10 mg/ml stock made up in water) were added and samples were incubated for 1 – 2 hours at 65ºC to remove proteins and RNA. DNA was purified by two phenol-chloroform extractions (using 500 µl phenol-chlorophorm), followed by a chloroform extraction to remove residual phenol and ethanol precipitation of DNA (as described in section 2.4.9 but without addition of sodium acetate). DNA pellets were air-dried and resuspended in 100 µl TE.

2.5.13 Isolation of total RNA from yeast

RNA was isolated from yeast for quantitative PCR for find out the expression of genes. Roche high pure RNA isolation kit was used. The yeast cells were harvested during mid-log phase at 3000 rpm for 5 min on a standard tabletop centrifuge. Supernatant was discarded and pellet was resuspended in 200 µl PBS. 10 µl of 0.5 mg/ml Zymolyase was added and incubated at 30ºC for 15 mins. Then 400 µl of lysis/binding buffer was added and vortexed for 15 seconds. The sample was then transferred to a high pure filter tube placed in a provided collection tube. The high pure filter tube assembly was then centrifuged again for 15 seconds. After centrifugation, the supernatant was discarded and 90 µl of DNase incubation buffer, 10 µl of DNase I were added to the upper reservoir of the filter tube and incubated at 20ºC for 15 mins. 500 µl of wash buffer I was added and centrifuged for 15 seconds. The flow through was discarded. 500 µl of wash buffer II was the added and centrifuged for 15 seconds. The filter tube was replaced back into the collection tube and centrifuged further for 2 more minutes to remove any residual wash buffer. The collection tube was then discarded and the filter tube was placed in a standard 1.5ml eppendorf. 50-100 µl of elution buffer was added to the upper reservoir to elute the RNA and centrifuged for 1min. The eluted RNA was used directly for RT-PCR or stored at -80ºC for further analysis.

2.6 Bioinformatics

2.6.1 Specialized computer programs

Table 6: Scientific programs used

Program	Application
Gene construction kit (GCK)	Plasmid map generation and *in silico* restriction
Sequencher 4.8	Sanger sequencing data analysis
Kaleida Graph	Plotting graphs
FlowJo V8.8.7	Flow cytometry analysis
MATLAB	Peak calling in genome-wide data sets
R program	Statistical analysis of data

2.6.2 Web tools

Genome-wide data sets, including primer and replication protein binding sites or replication timing were visualized on the UCSC genome browser (genome.ucsc.edu/). Furthermore, DNA sequences were submitted to the BLAT search option at the USCS genome browser to identify regions of high sequence similarity in the *S. cerevisiae* genome. Alternatively, Fungal BLAST on the *Saccharaomyces* Genome Database (SGD, www.yeastgenome.org) was used to identify similar sequences amongst yeasts other than *S. cerevisiae*. SGD was also the website predominantly used to retrieve information about yeast genes and their functions. The Gbrowse option on the resource browser (www.moseslab.csb.utoronto.ca/sarb/) was used to view genome sequence synteny between various strains of *Saccharomyces*. Primer sequences were designed using Primer3 Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and the yeast genome website (http://www.yeastgenome.org/cgi-bin/web-primer). Saccharomyces genome resequencing project (SGRP) websites (www.sanger.ac.uk/research/projects/ genomeinformatics/browser.html and www.moseslab.csb.utoronto.ca/sgrp/) were used to retrieve the sequence and genomic locations of SGRP plasmid library inserts and to retrieve SK1 genome sequence.

Table 7: Web tools used for this study

Program	Description
UCSC	Data visualization and BLAT genome search
SGD	Retrieval of general information about yeast
Primer3 Plus	Primer design
SGRP website	Retrieval of information about SGRP clones
Yeast genome website	Primer design

2.6.3 Unix based tools

BioScope 1.3.1 (LifeTechnologies) was used to map deep sequencing reads generated on an AB SOLiD machine to a reference genome. All subsequent analysis of deep sequencing data was done using Perl scripts written by Dr. C. Nieduszynski. First, BAM files, containing uniquely mapped sequencing reads, were converted to SAM files using the following command: samtools view -o name_of_sam_file.sam name_of_bam_file.bam chr_number. Second, the program 'sams_to_ratio.pl' was used to generate the ratio of the number of reads in each genomic window between two different datasets (command line: perl sams_to_ratio.pl control_sample_chr.sam experimental_sample_chr.sam window_size_in_bp > file_to_pipe_output_to.txt). Third, using the 'ratios_wig.pl' script all the ratio

files were converted and concatenated into a single wiggle file. The data from one or more wiggle files was plotted as an svg file with the svg_genome.pl script. A Fourier transformation, encoded as a phyton script written by Dr. A.P.S. de Moura, was applied to obtain smoothed replication timing profiles. Peak calling within genome-wide data was done using the 'peakdet' script for MATLAB, which was obtained from 'www.billauer.co.il/peakdet.html'. Sequence motif searches within peaks were done with a locally installed version of MEME (example command: 'nohup meme input_sequence.s -o /output_destination_folder -dna -mod zoops -nmotifs 10 -minw 10 -maxw 20 -revcomp -maxsize 300000 >myrun.out 2>myrun.err </dev/null&'). Intersections between datasets were performed using the program closestBed from BEDtools (example command: closestBed -a data_file1.bed -b data_file2.bed > output_file.txt').

2.6.4 Statistical Analysis

Spore viability data obtained (Section 3.2) was analyzed for statistical significance. Chi-square test is a statistical hypothesis test where the test is used to determine if there is a significant difference between the expected frequencies and observed frequencies in one or more categories. Data was compared for SMY037 (SK1 laboratory strain) & SMY084 (Dbf4-tag SK1), SMY086 (*ctf19Δ* SK1) & SMY143 (Dbf4-tag *ctf19Δ* SK1), SMY123 (*mad2Δ* SK1) & SMY141 (Dbf4-tag *mad2Δ* SK1). Standard error was calculated for the data obtained and p-value was calculated for each pair. P-value is a function of the observed statistics. The p-value is used in the context of null hypothesis testing in order to quantify the idea of statistical significance of evidence. The smaller the *p*-value, the larger the significance because it tells the investigator that the hypothesis under consideration may not adequately explain the observation. The P-value is the probability that a chi-square statistic having 2 degrees of freedom is more extreme than 19.58. We use the Chi-Square Distribution Calculator to find P= 0.0001. Interpret results. Since the P-value (0.0001) is less than the significance level (0.05), we cannot accept the null hypothesis.

3 Genome Replication in the meiotic proficient yeast strain SK1

3.1 Introduction

I have used the yeast strain SK1 to study pre-meiotic DNA replication in yeast. Typically, studies of meiosis and sporulation in yeast use the meiosis-proficient strain SK1 rather than the low meiosis efficiency strain S288c. After 24 h in sporulation medium, an S288c culture produced no mature asci, whereas 80% of cells in an SK1 culture sporulated (Deutschbauer and Davis, 2005). The phenotypic differences between sporulation in S288c and SK1 are not simply due to the kinetics of sporulation, as the efficiency of S288c does not exceed 20% even after one week in sporulation medium. Studies to find the differences between these strains mapped quantitative trait loci at single-nucleotide resolution to reveal that three genes *RME1, TAO3,* and *MKT1* (located on chromosomes 7, 9, and 14, respectively) directly contribute to the sporulation efficiency differences between the strains S288c and SK1 (Deutschbauer and Davis, 2005). Also strain SK1 has alleles with strong sporulation-promoting effects in the genes *SWS2* and *MKT1* whereas strain S288c has alleles of opposite effects in these genes. In the gene *SWS2*, the SK1 allele resulted in higher sporulation efficiency than the S288c allele. The gene *SWS2* has a major contribution to the difference between the two parental strains, as demonstrated by the efficient sporulation (~50%, determined after 48 h in liquid sporulation medium) of the S288c strain in which the two *SWS2* alleles were replaced by the corresponding alleles from the strain SK1. *MKT1*, the other gene with a SK1 promoting allele in this region seems to have a milder effect on sporulation efficiency (about 15% sporulation, determined also after 48 h in liquid sporulation medium (Ben-Ari et al., 2006)).

Centromeres play an essential role in proper chromosome segregation. The cells coordinate DNA replication and chromosome segregation to ensure faithful genome inheritance (Natsume et al., 2013). There are several genes, which are associated with cell cycle progression and DNA replication. Most of these genes have a significant role in cell cycle regulation. These genes have various functions during DNA synthesis, chromosome segregation, spindle pole body formation, sister chromatid separation etc., I have selected three genes *DBF4*, *CTF19* and *MAD2* which play an important role in mitotic chromosome replication and/or segregation.

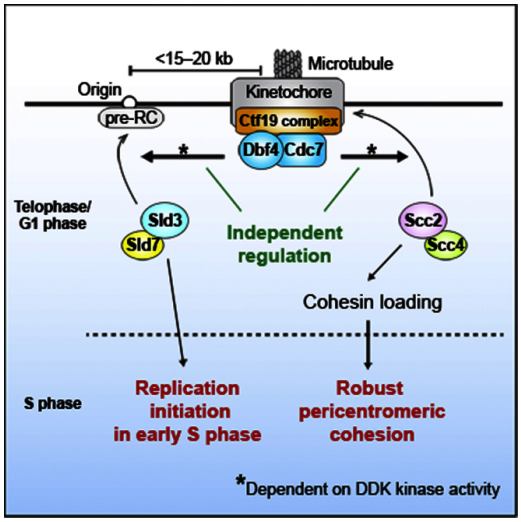


Fig 14: Cartoon showing the functions of Dbf4 and Ctf19. The CTF19 complex promotes the recruitment of *Dbf4* kinase on to the kinetochore which functions in promoting early S phase replication at this region. (Adapted from Natsume et al., 2013)

Centromeres replicate early during pre-mitotic S phase. The mechanism and physiological requirement for centromere replication was recently elucidated (Natsume et al., 2013) (Figure 14). According to the study, during pre-mitotic S phase, *ctf19Δ* and C-terminal tagging of Dbf4 delays centromere replication. Delay in centromere replication results in elevated chromosome loss (Figure 15). Indicated strains with the CFIII chromosome fragment were used for a chromosome loss assay. Loss of CFIII generated red sector in colonies. Error bars represent SD (Natsume et al., 2013). The chromosome loss rate was modestly enhanced with *DBF4-myc*, wherein DDK was reduced at kinetochores. Intriguingly, the chromosome loss frequency increased synergistically when *DBF4-myc* was combined with *mad2*D.

It is not known if the same equivalent mechanism operates during meiosis. If the same mechanism operates, then *ctf19Δ* and C-terminal *DBF4* tag are expected to delay centromere replication and elevate chromosome mis-segregation during meiosis. Chromosome mis-segregation during meiosis can be assayed by reduced spore viability. Therefore, to test the role of these genes during meiotic centromere replication, I assayed the spore viabilities of DBF4-*myc* tagged strain with a wild type strain, *mad2Δ* and *ctf19Δ* strains as controls.

Fig 15: Cartoon showing the chromosome loss rate during mitosis. Chromosome loss rate was modestly enhanced with DBF4-myc whereas the loss rate increased synergistically when DBF4-myc was combined with *mad2Δ*. The presence of a higher proportion of colonies with red sectors indicate high chromosome loss rate. (Adapted from (Natsume et al., 2013))

T

The *CTF19* gene (Systematic Name: *YPL018W*) encodes a kinetochore protein, which is needed for accurate chromosome segregation. Ctf19 plays an important role in mediating the attachment of centromere to the mitotic spindle by forming interactions between the microtubule-associated outer kinetochore proteins and the centromere-associated inner kinetochore proteins. Ctf19 contributes to establishing bipolar spindle-microtubule attachments and proper chromosome segregation. The CTF19 complex recruits the Dbf4 kinase on to the kinetochores, which leads to the early S phase replication of this region. This interaction also enhances the recruitment of Scc2-Scc4 to the centromeres, which promotes cohesin loading at pericentromeres (Natsume et al., 2013).

MAD2 (Systematic Name: *YJL030W*) is an essential mitotic spindle checkpoint gene. Mad2 plays a very important role in the metaphase to anaphase transition during mitotic division. It is the central component of the spindle assembly checkpoint (Fig 16). This prevents cells with incompletely assembled spindles from leaving metaphase, thus delaying anaphase. The progression of mitotic division from metaphase to anaphase is pronounced by the separation of sister chromatids. The checkpoint mechanism prevents the separation of sister chromatids and further transition into anaphase to safeguard the cell against any chromosome segregation errors. This spindle assembly checkpoint (SAC) delays anaphase until all sister chromatid pairs have become bipolarly attached (Zhou et al., 2002).

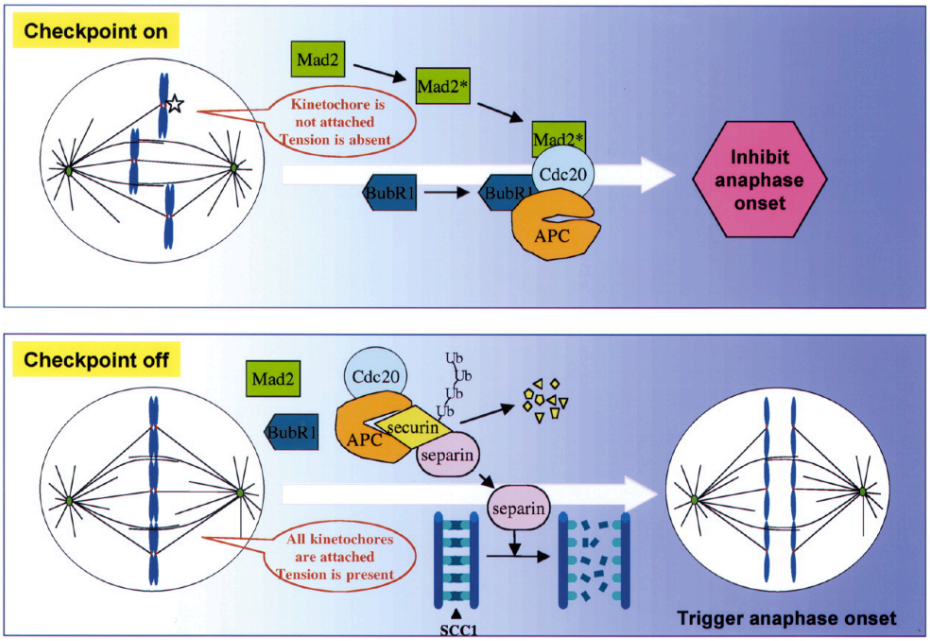


Fig 16: Spindle assembly checkpoint assembly. In the presence of unattached or improperly attached kinetochores, the spindle assembly checkpoint is switched on (upper panel). Unattached kinenetochores act as catalytic sites for the activation of Mad2. Activated Mad2 (Mad2*) then diffuses and prevents anaphase onset by inhibiting the activity of Cdc20-APC. After all the chromosomes are properly attached by kinetochore microtubules and aligned at the metaphase plate, the spindle assembly checkpoint is turned off (bottom panel). Mad2* is no longer generated, and BubR1 does not interact with Cdc20-APC, resulting in the activation of Cdc20-APC. (Adapted from (Zhou et al., 2002))

Previously a screen was carried out to find the genes required for meiotic cell cycle progression (Marston et al., 2004). They used a collection of *Saccharomyces cerevisiae* strains, in which the individual genes were deleted. Diploids were constructed that were homozygous and activity was analyzed through two meiotic events: sporulation efficiency and segregation pattern of a GFP marked chromosome (Marston and Amon, 2004). They constructed strains in which the genes *CTF19* and *MAD2* were deleted. Sporulation efficiency data of *ctf19Δ* showed that the mutants with *ctf19Δ* showed only 19% efficiency, indicating an impaired sporulation efficiency in *ctf19* mutants. *mad2Δ* mutants showed a sporulation efficiency of 52%, which halved the efficiency. The study suggests an important role for *CTF19* and *MAD2* in meiosis.

All the three genes *CTF19, DBF4* and *MAD2* play a very important role during mitosis, being required for stable chromosomal inheritance. However the activity of *DBF4* gene is still unclear. To study the activity of *DBF4* gene during meiosis, I have constructed a strain where the gene was tagged and observed the activity of strain during meiosis via spore viability, with *CTF19* and *MAD2* as controls.

3.2 Testing the genetic requirements of centromere replication during meiosis

I have used a spore viability assay to test the genetic requirements for stable chromosome inheritance. To study the difference between the wild type strain and the gene deletion/ gene tagged strains, I first looked at the spore viability of the wild type SK1 strain (SMY037). Wild type SK1 was grown on YPD agar and replica plated on to KAC-MIN agar to allow the cells to enter into meiosis. After 2 days of incubation, cells were examined under the microscope to check for sporulation (SK1 usually sporulates in two days). The cells were then removed from the plate and suspended in dissecting buffer containing zymolyase. Zymolyase is a yeast lytic enzyme, which dissolves the cell wall. The cells were then placed under the dissection microscope and haploid spores were separated from the tetrad and placed separately. The spores were incubated for 2-3 days to check for viability on the basis of colony growth.

After 2 days of incubation, the plate was examined (Figure 17a) and colonies were counted for spore viability. The number of visible viable spores (viable colonies) was counted against the total number of spores to calculate spore viability of the strain. The spore viability for wild type SK1 strain is 97%. Viability was calculated from a total of 96 tetrads dissected, which would give 384 spores in total. However the total viable spores observed were 372, making SK1 spores 97% viable. Below is a pie-chart representation of viability of wild type SK1 strain (Figure 17b). Initially a set of 48 tetrads was dissected to look at viability. Later to see that the results were consistent through out, the number of tetrads dissected was increased and the results were found to be consistent with the initial result.

a) b)

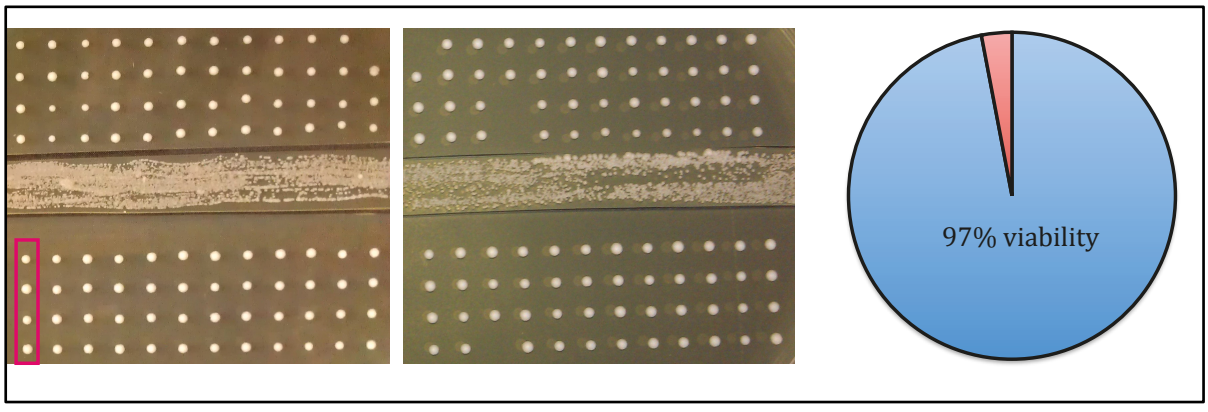
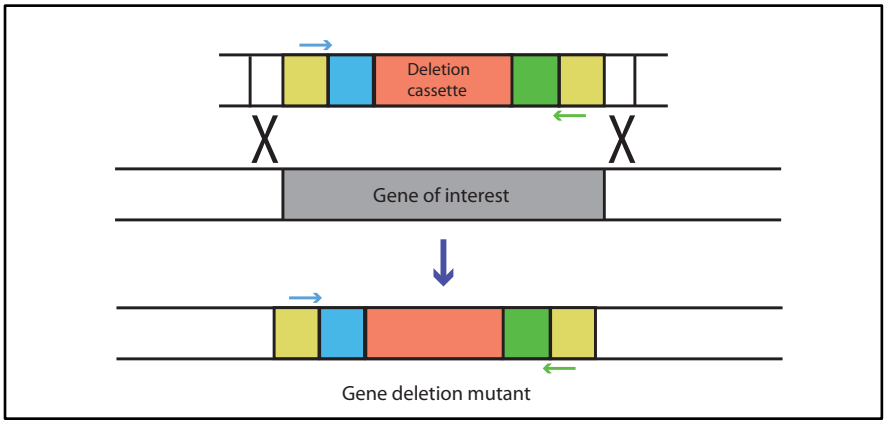


Figure 17: a. Spore viability of wild type SK1 strain. The spore viability of wild type SK1 strain (SMY037) was tested through tetrad dissections. Tetrad dissection is a process where the sporulating cells were treated with Zymolyase and individual cells are carefully moved to gridded positions. b. Pie chart representation of spore viability of SK1 strain. Blue area indicates the viability of the strain (97%). SK1 wild type strain used was SMY037

To study meiosis in the sporulation proficient SK1 strain background, I deleted or tagged genes of interest. For gene deletions, I amplified a gene deletion construct (by PCR) from previously constructed yeast deletion strains. For gene tagging, I amplified a gene-tagging construct (by PCR) from existing W303 strains in which the gene of interest was already tagged. Amplified cassettes were transformed into haploid SK1 strains and successful integrants were selected for. For meiotic studies, I require homozygous diploid strains where the gene of interest has been deleted or tagged from both the alleles; hence the transformed haploid strains were either mated to obtain a diploid strain or diploids produced by transient expression of the *HO* gene. Gene deletion was confirmed via PCR.



F **Fig 18: General strategy for gene deletion strain construction.** A PCR based strategy was used to create gene deletion strains. The deletion cassette was amplified using a pair of flanking primers. The amplified product homologously recombines to replace the gene of interest with the deletion cassette. The replacement of the gene with the deletion cassette was verified by PCRs spanning the whole gene, spanning the left junction or the right junctions.

Figure 18 shows the general strategy of gene deletion strain construction I have used to construct my strains. The area in red indicates the deletion cassette that was amplified and lifted from the S288c deletion library. Two specific primers (in blue and green) were designed to amplify the deletion cassette with regions of flanking homology to target integration. Diploid yeast deletion strains were constructed that were homozygous for a particular deletion. The new SK1 strains were genotyped by PCR. The diploid homozygous strains were tested for faithful meiosis through a spore viability assay. Cells were allowed to enter into meiosis and the tetrads were dissected using a dissection microscope. The viability of spores provides an indirect measure of faithful chromosome inheritance during meiotic division.

3.2.1 DBF4 tagging

To investigate whether C-terminal tagging of Dbf4 adversely affects meiosis, I created a homozygous diploid DBF4 tagged strain. In the lab we have a strain (T9394) where Dbf4 is C-terminally tagged with 9 Myc epitopes linked to a selectable marker in the W303 background (Natsume et al., 2013). However, to study meiosis, I need a strain in SK1 background. I amplified the *DBF4* tagging cassette from T9394 using primers CN224 and SM019. The amplified tagging cassette was transformed into haploid SK1 strains SMY035 (mat α) and SMY036 (mat a). The resulting two haploid SK1 strains, in which *DBF4* was tagged, were mated together to produce a diploid strain. The ploidy of the strain was confirmed via PCR with primers CA377, CA378 and CA379. Appearance of two bands on a gel picture indicates a diploid strain (Fig: 19c). To confirm successful tagging of the *DBF4* gene, a PCR was performed with CN2130 and SM021 which confirms the *MYC-DBF4* junction, giving a ~1.2kb band (Figure 19d).

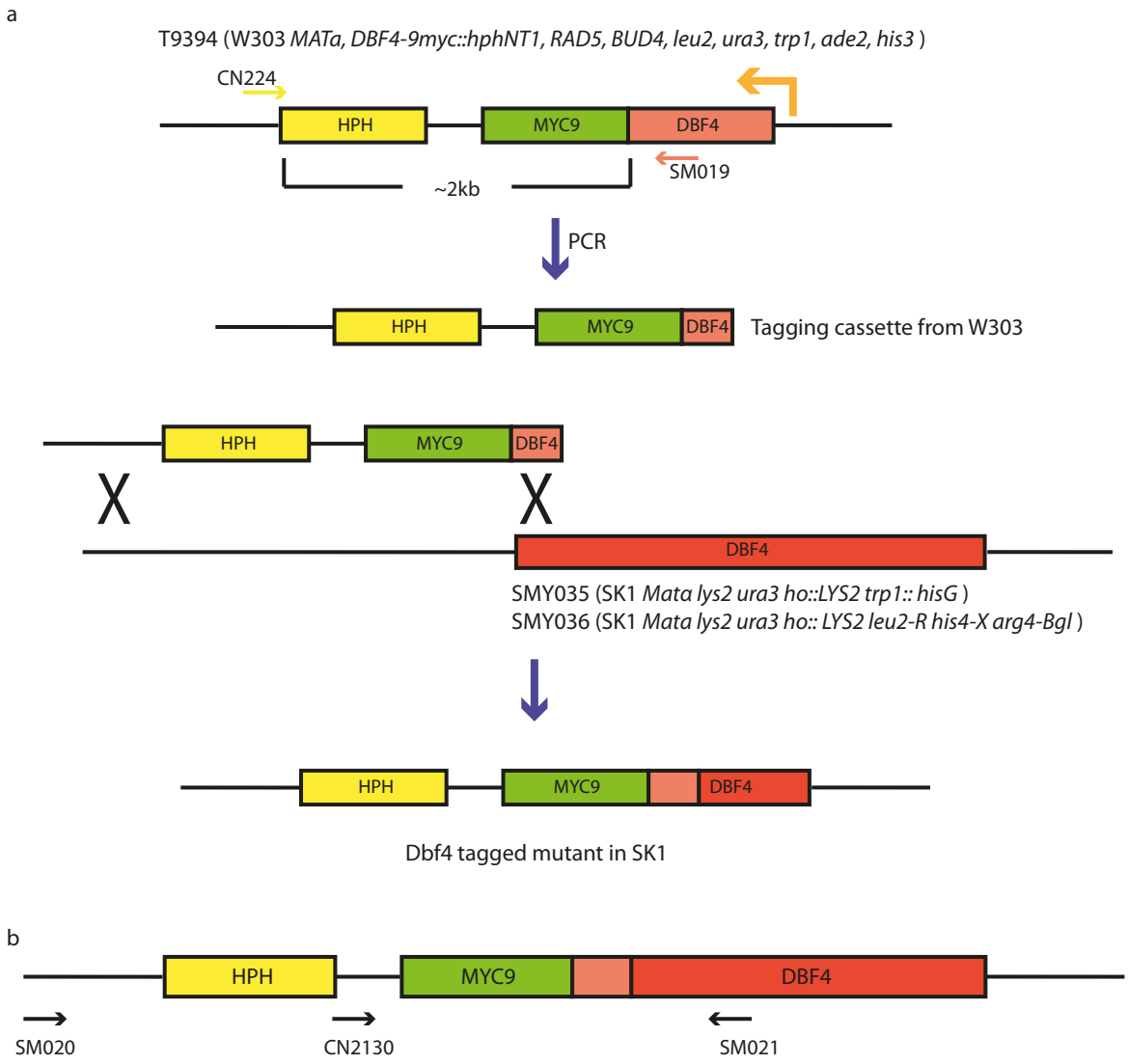


Figure 19: a. DBF4 tagged SK1 strain construction. *MYC* tagged *dbf4* gene from a W303 strain (T9394) is amplified with a HPH selectable marker using primers CN224 and SM019. The site of the amplified product is confirmed by PCR. The *dbf4-myc* tagging construct was introduced into the haploid SK1 yeast cells by a standard yeast transformation protocol. The resultant transformants were tested for integration by colony PCR. The haploid cells confirmed for dbf4 tagging were crossed to produce a diploid SK1 strain. b. Confirmation of dbf4 tagging. To confirm successful tagging of *DBF4* gene, various PCRs were performed. PCR with primers SM021 and CN2130 confirmed the *MYC-DBF4* junction.

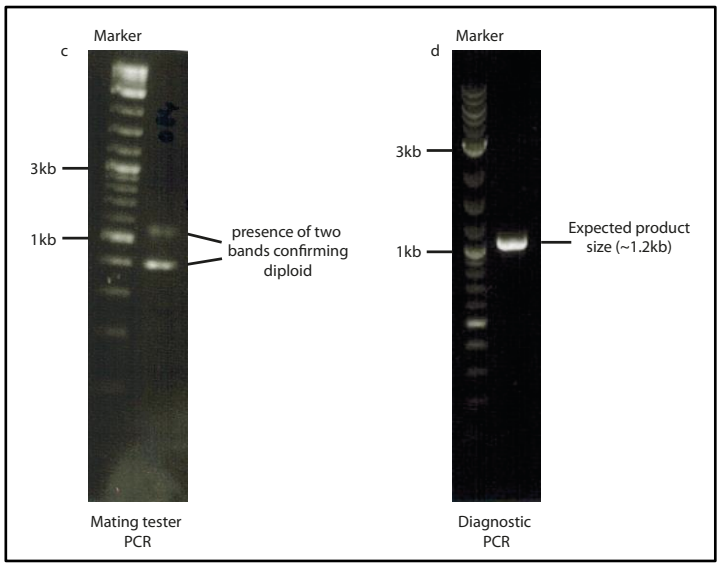


Figure 19: c. Confirmation of mating type of *dbf4* tagged diploid SK1 strain.

d. Confirmation of *Myc* tag with primers SM021 and CN2130.

Once the strain was confirmed, the DBF4 tagged SK1 strain was allowed to sporulate by growing on minimal medium. It took 2-3 days for the strain to sporulate and the tetrads were observed under the microscope. After confirming sporulation, tetrads were dissected and incubated at 30ºC to allow colonies to form. After 2 days of incubation, the plate was examined (Figure 20a) and the number of visible spores was counted against the total number of possible spores to calculate the spore viability of the strain. The spore viability of a *DBF4* tagged SK1 strain is 87% (Figure 20b). Viability was calculated from a total of 96 tetrads dissected, which would give 384 spores in total. However the total viable spores observed were 333, making SK1 strain 87% viable.

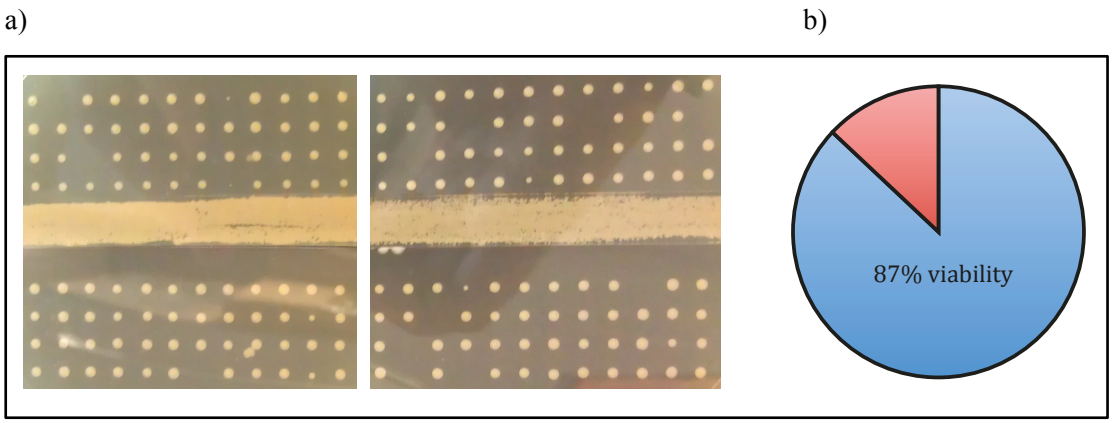


Figure 20: a. Spore viability of *DBF4* tagged SK1 strain. Spore viability of *dbf4* tagged SK1 strain was tested through tetrad dissections. b. Pie chart representation of spore viability of *DBF4* tagged SK1 strain. Blue area indicates the viability of the strain SMY084 (87%).

3.2.2 *CTF19* deletion

To investigate the meiotic ability of a *ctf19* deleted SK1 strain, I used strain T9791, a kind gift from Tomo Tanaka (University of Dundee), in which *CTF19* was deleted with the *KanMX* cassette (Natsume et al., 2013). The deletion cassette was amplified using primers SM015 and SM016 via polymerase chain reaction (Natsume et al., 2013). The amplified deletion cassette was transformed into haploid SK1 strains SMY035 and SMY036 (Figure 21a). The two haploid SK1 strains in which *CTF19* was deleted were mated to produce a diploid homozygous *ctf19Δ* strain.

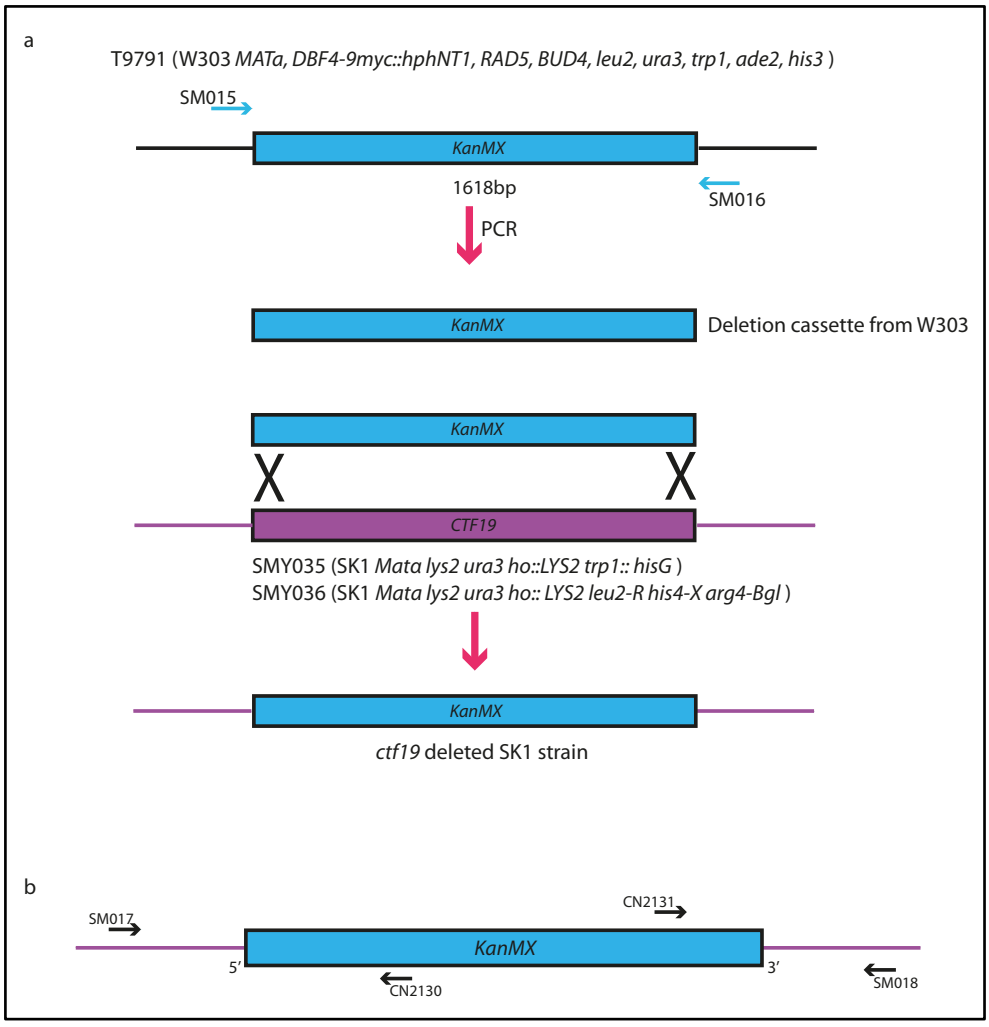


Figure 21a: ***CTF19* deletion SK1 strain construction.** *KanMX* cassette from a W303 strain (T9791) was amplified using primers SM015 and SM016. The size of the amplified product was confirmed by gel electrophoresis. The *KanMX* cassette was introduced into the haploid SK1 yeast cells by standard yeast transformation. The resultant transformants were tested for successful recombination by colony PCR. The haploid cells confirmed for *ctf19* deletion were crossed to produce a diploid SK1 strain. **8b: Confirmation of ctf19 deletion.** To confirm successful deletion of *ctf19* gene, various PCRs were performed. PCR1 with primers SM017 and CN2130 to confirm the 5' junction, PCR2 with primers CN2131 and SM018 to confirm the 3' junction and a spanning PCR with primers SM017 and SM018 to confirm the insertion of the entire deletion cassette.

To confirm successful deletion of the *CTF19* gene, PCRs was performed with CN2130 and SM017 to confirm the 5' junction (PCR1, ~500bp), with CN2131 and SM018 to confirm the 3' junction (PCR2, ~900bp) and a spanning PCR with SM017 and SM018 to confirm the insertion of the entire deletion cassette (PCR3, ~2kb) (Figure 21c). Confirmation PCRs of *ctf19* deletion showed expected band sizes. The strain was then allowed to sporulate and tetrads were dissected following treatment with Zymolyase. After 2 days incubation of dissected tetrads, the plates were observed and found that the spore viability of *ctf19* deleted SK1 diploid is 0% (Figure 21d). Viability was calculated from a total of 20 tetrads dissected, which would give 80 spores in total. But none of those spores grew, indicating no spore viability i.e 0%. However due to the low viability observed in *ctf19* deletion strain, few tetrads were dissected to test viability.

c) d)

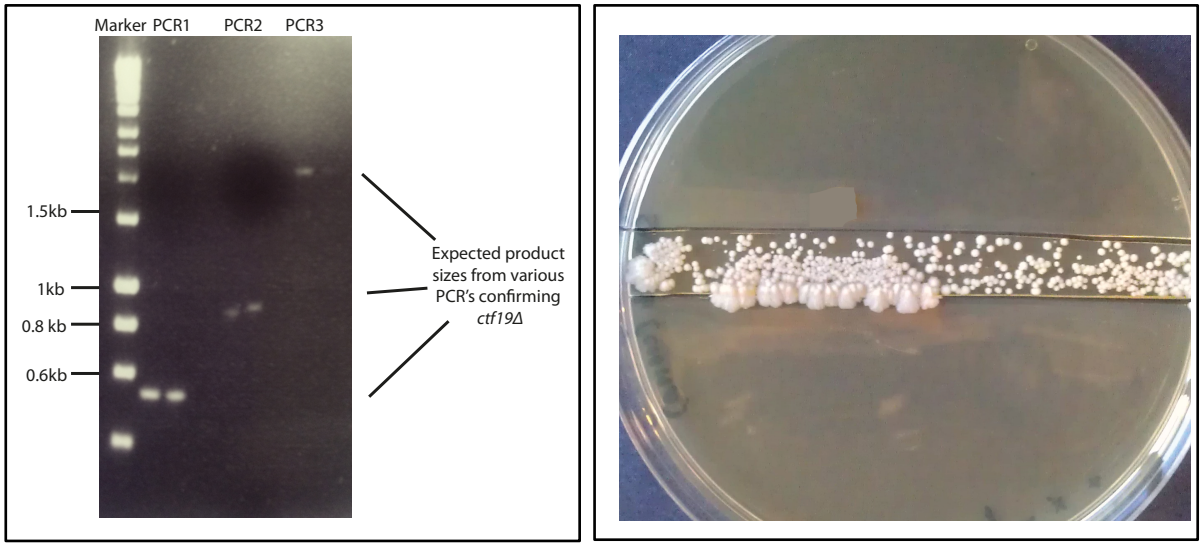


Figure 21c. Confirmation of *ctf19* deletion. The deletion of *ctf19* gene was confirmation by performing PCRs with various primer combinations to prove deletion at 5' junction, 3' junction and the entire insertion of the deletion cassette. **d. Spore viability of *ctf19* deleted SK1 strain** The spore viability of *ctf19* deleted SK1 strain was tested through tetrad dissections and was found to have 0% spore viability.

3.2.3 *MAD2* deletion

To investigate the meiotic requirement for *MAD2,* I deleted the *MAD2* gene in the SK1 strain background. The strain in which *MAD2* was replaced with *KanMX* cassette was identified from the S288c yeast deletion library and the deletion cassette was amplified using primers SM042 and SM045 via polymerase chain reaction. The amplified deletion cassette was transformed into haploid SK1 strains SMY035 and SMY036 (Figure 22). The two haploid SK1 strains in which *MAD2* was deleted were mated to produce a homozygous diploid *mad2Δ* strain.

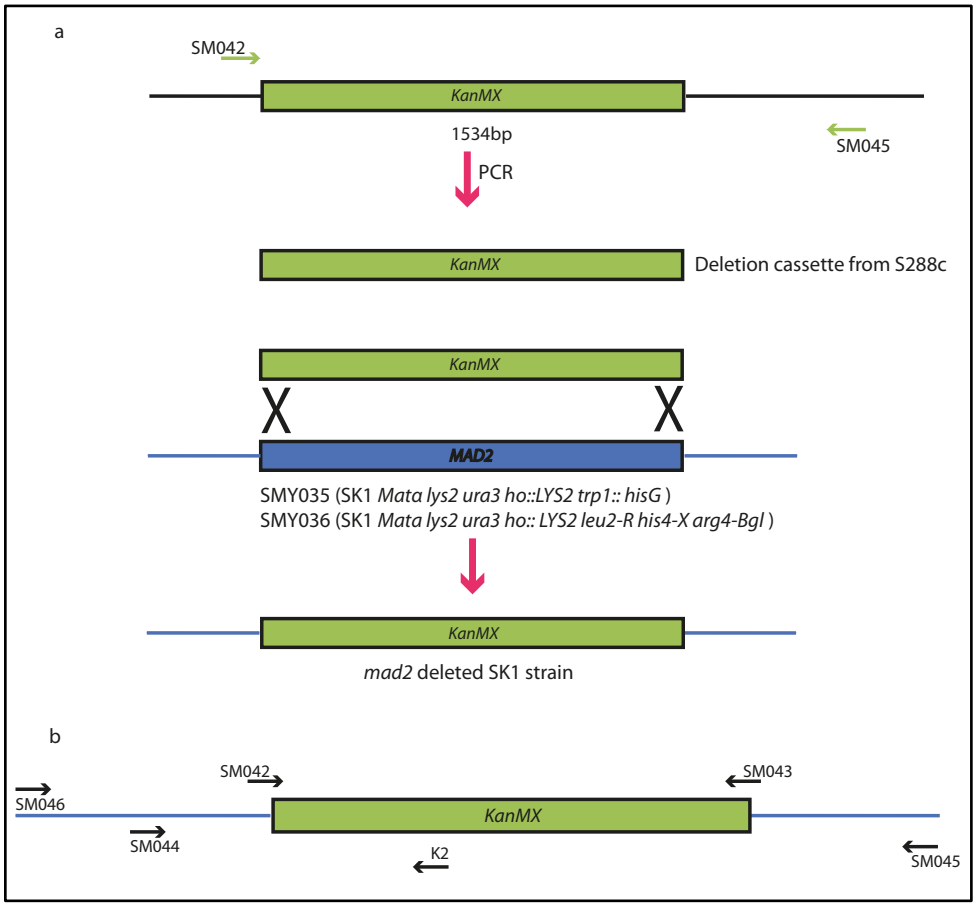


Figure 22a: *MAD2* deletion SK1 strain construction. *KanMX* cassette from the yeast gene deletion library (YJL030W) was amplified using primers SM042 and SM043. The size of the amplified product was confirmed by gel electrophoresis. The *KanMX* cassette was introduced into the haploid SK1 yeast cells by standard yeast transformation. The resultant transformants were tested for insertion by colony PCR. The haploid cells confirmed for *mad2* deletion were crossed to produce a diploid SK1 strain. **b. Confirmation of *mad2* deletion.** To confirm successful deletion of *mad2* various PCRs were performed. PCR1 with primers CA377, CA378 and CA379 to confirm the mating type of the strain, PCR2 and PCR3 with primers SM044 and K2, SM046 and K2 respectively to confirm *mad2* deletion at the 5' junction.

To confirm successful deletion of the *MAD2* gene, various PCRs were performed. PCRs with CA377, CA378 and CA379 were performed to confirm the mating type of the strain (Figure 22c, lane 1). Another set of PCRs was done with primer combinations SM044 - K2 and SM046 - K2 to confirm the deletion of *mad2* at the 5' junction and was confirmed showing the expected band sizes. The strain was then allowed to sporulate and tetrads were dissected. After 2 days incubation of dissected tetrads, the plates were observed and the spore viability of *mad2*Δ diploid was found to be 26% (Figure 23). Viability was calculated from a total of 24 tetrads dissected, which would give 96 spores in total. However the total viable spores observed were only 25, making *mad2*Δ SK1 strain 26% viable.

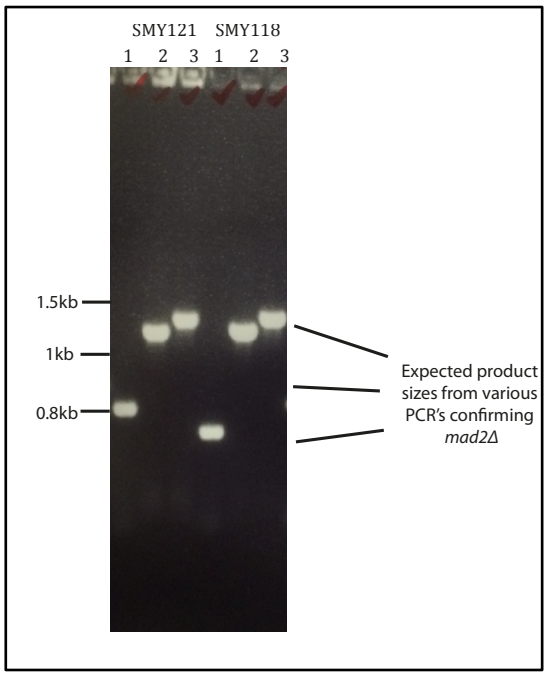


Figure 22c: Confirmation of *mad2* deletion. To confirm successful deletion of *mad2* various PCRs were performed. SMY121 and SMY118 are the two haploid SK1 strains where *mad2* gene has been deleted. PCR1 was with primers CA377, CA378 and CA379 to confirm the mating type of the strain, PCR2 and PCR3 with primers SM044 and K2, SM046 and K2 respectively to confirm the *mad2*Δ at the 5' junction. Expected band sizes confirmed the deletion of *mad2*Δ.

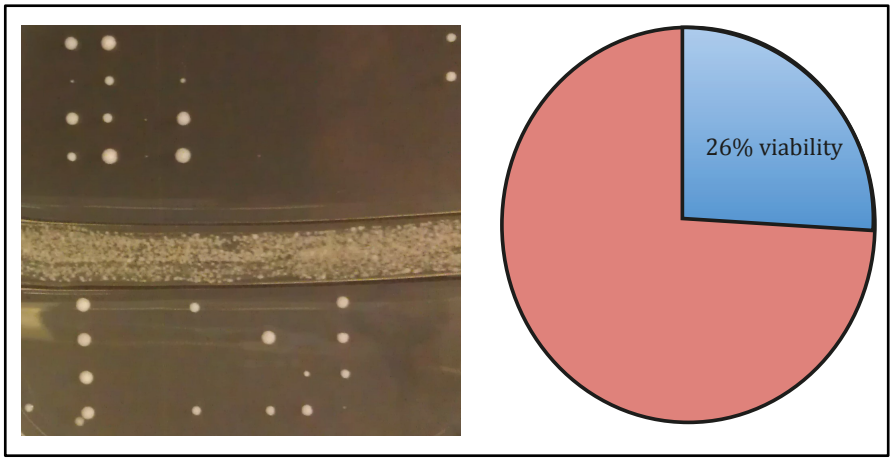


Figure 23: Spore viability of *mad2* deleted SK1 strain. Spore viability of *mad2* deleted SK1 strain was tested through tetrad dissections. Pie chart representation of spore viability of *mad2* deleted SK1 strain. Blue area indicates the viability of the strain SMY121 (26%).

3.2.4 Spore viabilities of double deletion and tagging strains

Double deletion and tagging strains were constructed from the earlier constructed single gene deletion strains. The haploid single gene deletion or tagged strains were crossed with each other to generate a diploid strain with double deletions. The obtained heterozygous diploid strain was made to sporulate and each of the spore was tested for both *mad2Δ* and Dbf4-tag. A spore positive for both *mad2Δ* and Dbf4-tag was taken and the diploid of the same phenotype was made by the transient

expression of HO gene. *mad2Δ* SK1 Matα strain (SMY118) which was constructed by single deletions (section 3.2.3) was crossed with Dbf4-tag tagged SK1 Mata strain (SMY073) (section 3.2.4.1) (Figure 24). Recombinants were allowed to sporulate and tetrads were dissected to produce spores. Spores were tested for *mad2Δ* and Dbf4-tag. Individual haploid colonies, which were positive for both *mad2Δ* and Dbf4-tag were selected and the diploid of the same phenotype was made by the transient expression of *HO* gene. To confirm the *mad2* deletion and Dbf4 tagging in the resulting strain, a set of PCRs were performed. SMY141 is the SK1 diploid strain with *mad2Δ* and Dbf4-tag. The diploid mating type of the strain, *mad2Δ* and Dbf4-tag were tested and confirmed by PCR (Figure 25)

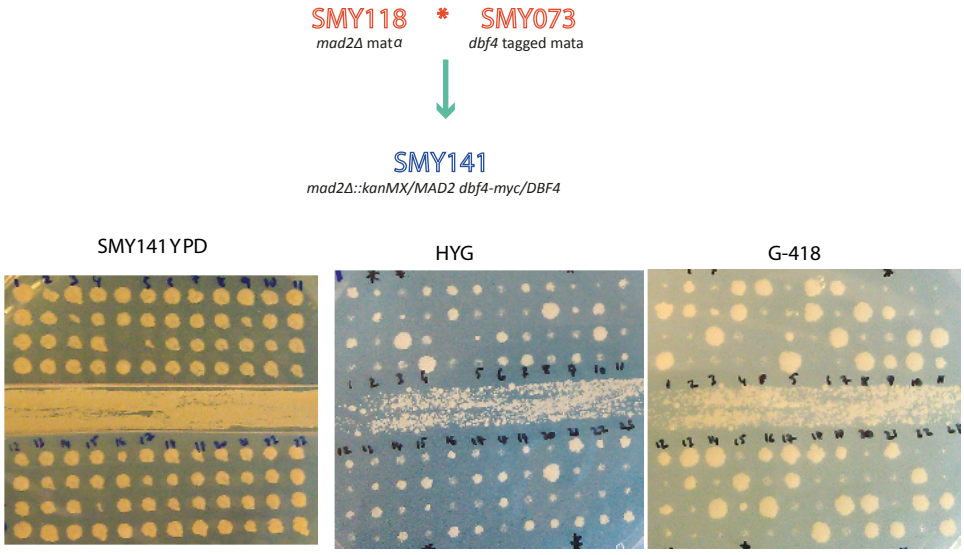


Figure 24: Construction of *mad2, dbf4* double deletion strains. Double deletion strains were constructed by crossing the single deletion strains. Strains were genotyped by growing them on selective media.

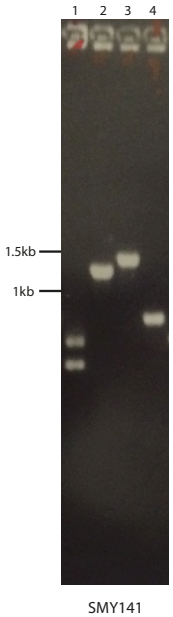


Figure 25: PCR confirmation of genotype. Various sets of PCRs were performed to confirm *mad2* deletion and *dbf4* tagging in the double deletion strain. The resultant homozygous diploid is SMY141. Lanes 1 confirms the diploid mating type of the strain, giving two bands on PCR with CA377, CA378 and CA379. Lanes 2 and 3 were the PCRs with primers SM044-K2 and SM046-K2 to confirm *mad2Δ* at the 5' junction, with an expected band size of ~1.5kb. Lane 4 confirms the *dbf4-tag* at the MYC junction giving a band size of ~800bp with primers SM020 and SM021.

The spore viability of the *mad2Δ dbf4* tagged homozygous diploid strain, was checked in a similar manner as before. The dissected tetrads were incubated again for 2 days to check for spore viability. The spore viability of the double deletion strain was found to be 10% (Figure 26). Viability was calculated from a total of 36 tetrads dissected, which would give 144 spores in total. However the total viable spores observed were only 15, making the spores 10% viable.

Figure 26: a. Spore viability of *mad2* deleted *dbf4* tagged SK1 strain. The spore viability of double deletion SK1 strain was tested through tetrad dissections. **b. Pie chart representation of spore viability of double deletion SK1 strain.** Blue area indicates the viability of the strain SMY141 (10%).

c*tf19Δ dbf4* tagged strains were constructed from the earlier constructed *ctf19Δ* and *dbf4* tagged SK1 haploid cells and crossed to get a diploid with double deletion, in the same way as the previous double deletion strain (*mad2Δ dbf4* tagged) (Figure 27). SMY079 is a *ctf19Δ* Mat α strain and SMY076 is a *dbf4* tagged Mat a strain. These strains were crossed to produce a diploid strain with double deletions. The obtained heterozygous diploid strain was made to sporulate and the each of the spore tested for both *ctf19Δ* and *dbf4-tag*. A spore positive for both *ctf19Δ* and *dbf4-tag* was taken and the diploid of the same phenotype was made by the transient expression of *HO* gene. SMY143 is the SK1 strain with *ctf19Δ* and *dbf4* tag. The diploid mating type of the strain, *ctf19Δ* and *dbf4* tag were confirmed by PCR (Figure 28).

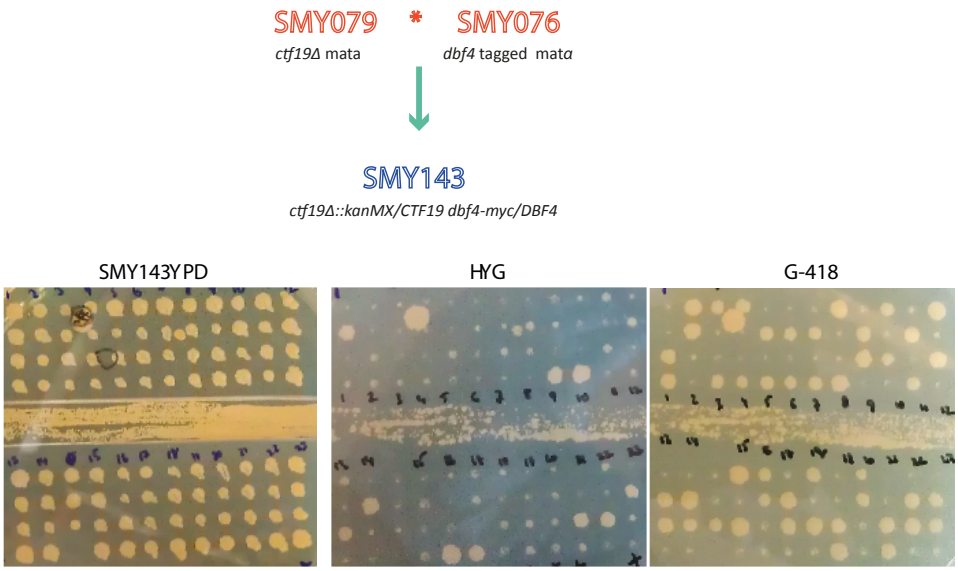


Figure 27: Construction of *ctf19, dbf4* double deletion strains. Double deletion strains were constructed by crossing the single deletion strains. Strains were genotyped by growing them on selective media.

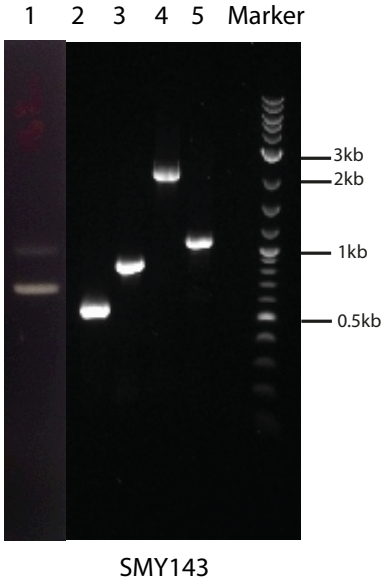


Figure 28. PCR confirmation of genotype. SMY143 is the *ctf19Δ dbf4*-tag strain. Lane 1 confirms the diploid mating type of the strain (PCR with primers CA377, CA378 and CA379). Lane 2 is the spanning PCR with primers SM020 and SM021 to confirm *dbf4* tagging. Lane 3 confirms the *dbf4* tagging at the 5' junction with primers SM021 and CN2130. Lanes 4 and 5 confirms the spanning *ctf19Δ* and *ctf19Δ* at the 3' junction with primers SM017 - SM018 and SM018 – CN2131 primer combinations.

The spore viability of the *ctf19Δ dbf4* tagged strain, was checked as before. The dissected tetrads were incubated for 2 days to check for spore viability. The spore viability of the double deletion strain was found to be 17%. Viability was calculated from a total of 48 tetrads dissected,

which would give 192 spores in total. However the total viable spores observed were only 33, making the strain 17% viable.

a) b)

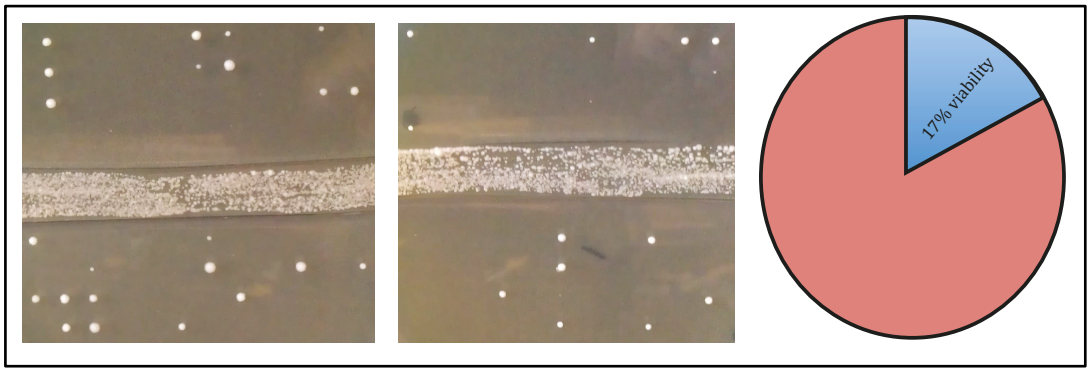


Figure 29: a. Spore viability of *ctf19* deleted *dbf4* tagged SK1 strain. The spore viability of homozygous diploid SK1 strain was tested through tetrad dissections. b. Pie chart representation of spore viability of double deletion SK1 strain. Blue area indicates the viability of the strain SMY143 (17%).

Table 8: Spore viabilities of wild type SK1 strain and other gene deletion strain

Strain (genotype)	Total tetrads	Total spores	Viable spores	% Viability
SMY037 (SK1 wild type)	96	384	372	97%
SMY084 (*DBF4* tagged SK1)	96	384	333	87%
SMY086 (*ctf19* Δ SK1)	20	80	0	0%
SMY123 (*mad2* Δ SK1)	24	96	25	26%
SMY141 (*mad2* Δ *DBF4* tagged SK1)	36	144	15	10%
SMY143 (*ctf19* Δ *DBF4* tagged SK1)	48	192	33	17%

Table 8 gives a summary of spore viabilities for the indicated genotypes. Wild type SK1 strain spore viability is high. Tagging DBF4 reduced the spore viability of the SK1 slightly, however the difference is only just statistically significant (Figure 30). However, deletions of *mad2* and *ctf19* genes greatly reduced the spore viability of the strain, indicating the functionality of these genes during meiosis. *ctf19*Δ SK1 strain showed no spore viability, indicating that *CTF19* plays an essential role during meiosis. The result is in agreement with a previous study, which showed less than 1% spore viability of strains with *ctf19* gene deletions (Rabitsch et al., 2001). Another study has identified 19%

spore viability for a strain with *ctf19*Δ (Marston et al., 2004). Interestingly in the gene deletion and gene tagged strains, the high viability of only *Dbf4* tagged strain has been considerably reduced by the additional deletion of *mad2* and *ctf19* genes.

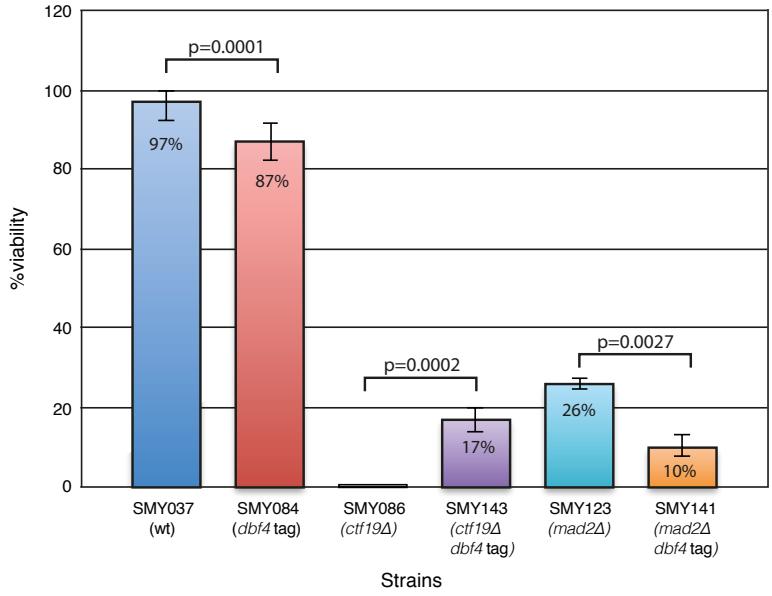


Figure 30: Comparison of spore viabilities of various strains. Data from the spore viability assay is summarized. Data was evaluated to test the statistical significance.

Wild type, *dbf4-myc* tag, *ctf19Δ* and *mad2Δ* strains were compared using the data obtained from spore viability assay (Figure 30). Viability of Dbf4-*myc* tag strain was reduced to 87% when compared to the viability of wild type SK1. The difference in viability between the two strains was found to be statistically significant using a chi-square test ($p = 0.0001$). This indicates that a C-terminal tag on DBF4 disrupts its function during meiosis, resulting in the reduced spore viability. *ctf19Δ* and *mad2Δ* strains were used as controls to assess the activity of Dbf4. It was earlier reported that *CTF19* plays an important role during meiosis and *ctf19Δ* mutants impair the sporulation efficiency of the cell (Marston et al., 2004). Data obtained for *ctf19Δ* mutants showed 0% viability and due to the low viability of the strain, very few tetrads were dissected (20 tetrads dissected). Surprisingly, Dbf4-*myc* tag partially rescued the *ctf19Δ* spore viability (bar 4 in Figure 30) and the reason for this is unknown. More investigation is necessary to determine how and why Dbf4-*myc* tag has partially rescued viability in a *ctf19Δ*. *mad2Δ* mutant was tested for spore viability in comparison with the Dbf4-*myc* tag. *mad2Δ* mutant showed only 26% viability and the viability of a *mad2Δ* mutant was further reduced on addition of a *dbf4-myc* tag to the strain. The data suggests the role of *DBF4* gene in meiotic replication. These data are consistent with a role for *DBF4*, during meiosis that gives an increased requirement for the spindle assembly checkpoint. However, the spore viability assay doesn't offer a detailed way to look at the role of *DBF4* during meiosis. Gene replacement from a

tagged gene of a different strain may also have changed the allele of the modified gene, hence might be a reason for loss of spore viability of the strain. Hence, I sought to establish a genome wide approach to obtain the mitotic and meiotic DNA replication data.

3.3 Mitotic vs. Meiotic SK1 genome wide replication profiles

Initially, I have used a spore viability assay to look at the genetic requirement of yeast strain SK1 during meiosis and to compare it with mitotic requirement. However, the assay doesn't offer a mechanistic explanation for the differences in spore viability. At the time of undertaking this work, there was no established assay for looking genome wide at pre-meiotic DNA replication. Therefore, I aimed to establish an assay that could measure pre-meiotic DNA replication. I used the genome wide replication approach, which uses fluorescence activated cell sorting (FACS) and deep sequencing to produce genome wide replication profiles.

3.3.1 Mitotic replication profile for SK1

Whole genome replication of SK1 strain during mitosis was examined through deep sequencing. Deep sequencing measures the DNA copy number in replicating cells compared to non-replicating cells. The sort-seq approach uses both deep sequencing and flow cytometry to study the replication dynamics genome wide (Muller et al., 2014). Cells were allowed to grow at 30ºC to reach the exponential phase. Washed cells were fixed with 70% ethanol and washed with Sodium Citrate, sonicated and treated with RNase A and proteinase K. Washed and pelleted cells were resuspended in nucleic acid stain SYTOX. Replicating (S-phase) cells were then enriched using fluorescence activated cells sorting (FACS). Stationary phase cells were also collected as a non-replicating control. The purity of the sorted cell fractions was confirmed by flow cytometry.

DNA was extracted from sorted cells and stationary phase cells. DNA was then sent for Illumina deep sequencing (50bp paired-end reads). Reads were generated for each samples for sorted mitotic, meiotic and stationary phase cells (Table 9). The reads generated were aligned to reference *Saccharomyces cerevisiae* (S288c) genome (sacCer3).

Table 9: Total number of reads generated for each sample during deep sequencing

Cells	No. Of reads generated
Sorted mitotic SK1 cells	36866280
Stationary cells	39068403
Sorted meiotic SK1 cells	32395467

Deep sequencing gives a precise measure of DNA copy number in replicating cells relative to the non-replicating cells and can detect the differences between pre-replicative (1 copy) and post replicative (2 copies) per cell. Regions of the genome that replicate early in S phase will be present at two copies in virtually all of the replicating (S-phase) samples. By contrast, late replicating regions will be present at one copy in a majority of the replicating sample. Therefore comparison of DNA copy number between a replicating and non-replicating (control) sample allows measurement of the relative replication time. The ratio of sequencing reads was calculated in each 1kb window between the replicating and non-replicating samples. All the ratios were then divided by an empirically determined factor such that the lowest value becomes 1. Then we find maximum (as expected) is 2.0 or less.

Genome wide replication data of SK1 strain obtained from this study is shown in Figure 31. The profiles obtained generally resemble published yeast replication profile data. This suggests that the procedure we followed in obtaining the replication profiles worked well. Peaks represent the location of replication origins, which are marked by vertical lines. In the figure Y-axis represents the relative copy number and X-axis indicate the chromosome coordinates (bp). A copy number of 2 indicates that the origin is highly active. The higher the copy number, the more active the origin is. Centromeres are the regions on the chromosome where the replication origins initiate first; they are the early replication sites. Moving away from the centromere, origins begin to replicate late and the telomere regions are the later replicating regions.

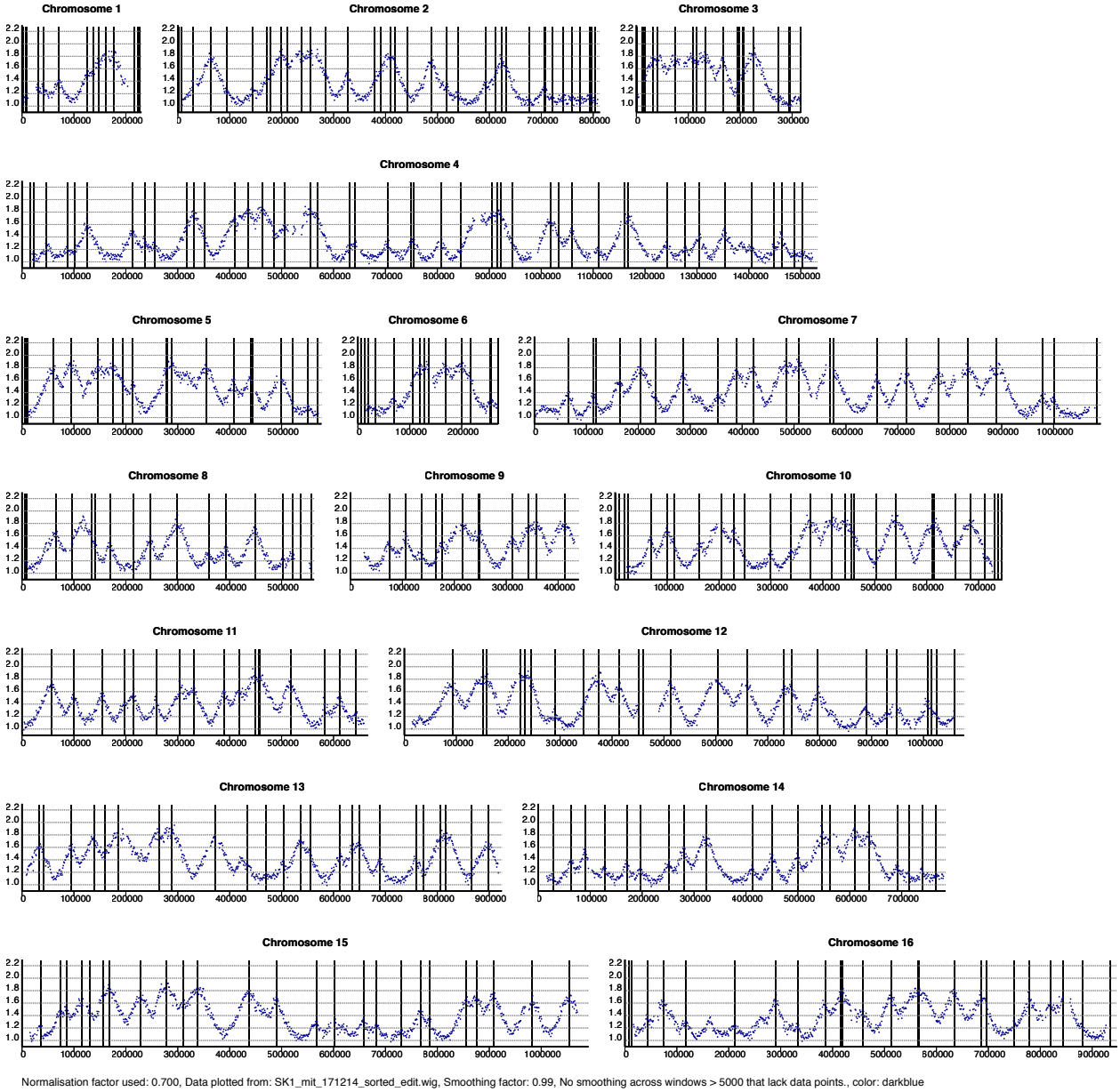


Figure 31: Genome wide replication profile of mitotic SK1 strain. Replication profiles for the 16 chromosomes of SK1 strain. X-axis indicates the chromosome coordinates (bp) and y-axis represents the relative copy number. Black vertical lines indicate the location of confirmed replication origins.

3.3.2 Comparison of replication profiles between SK1 and W303 strains

Different yeast strains could differ in their replication in various aspects. W303 is the most commonly used strain for laboratory studies. I compared the replication profiles of the SK1 and W303 strains. The SK1 mitotic replication profile was compared with the same resolution W303 genome-wide replication profile data from our lab (published (Muller et al., 2014)). There are some differences apparent in the activity of origins, for example their time of activation etc. To study the differences, both the replication profiles were plotted on the same axis one against another. Blue plot indicates the SK1 replication profile, whereas red plot indicates the W303 profile. Careful comparison of both profiles indicates that the two profiles are virtually identical with only very minor differences (Figure 32).

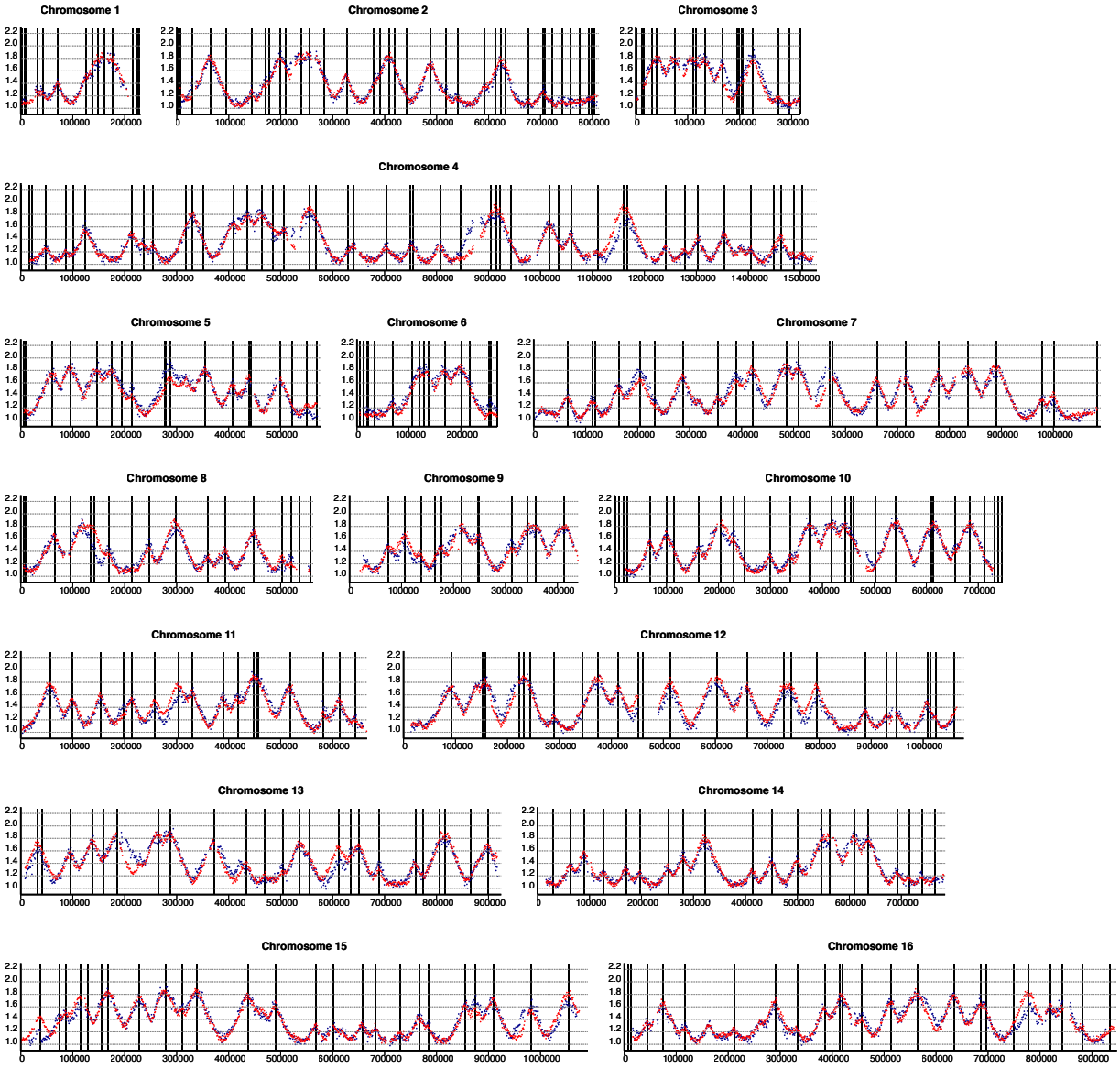


Figure 32: Comparison of replication profiles of W303 and SK1 strains. SK1 profile was indicated in blue whereas the W303 profile in red. The vertical lines indicate origins of replication. X-axis indicates the chromosome coordinates (bp) and the y-axis indicate the relative copy number.

The differences found between the two-replication profiles were assessed in detail. Major differences were listed (Table 10) and plotted for detailed analysis (Figure 33). The figure shows the six chromosomes in which the differences were observed. Vertical bars represent the location of experimentally validated replication origins. Selected origins were named above the plots.

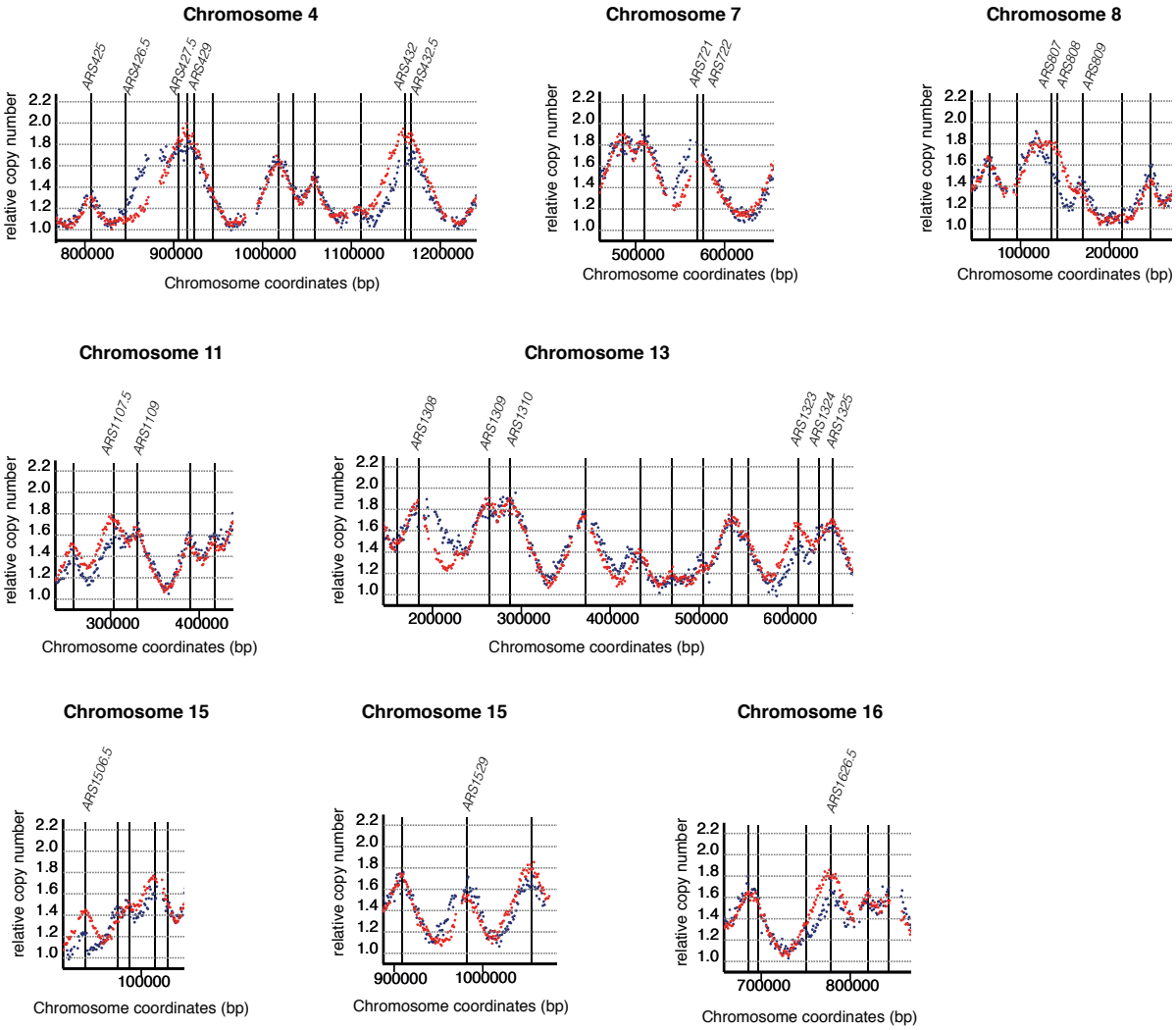


Figure 33: Various differences between SK1 and W303 replication profiles. Blue indicates SK1 strain and red indicates W303.

Interestingly, careful analysis of differences between SK1 and W303 profiles showed that most of the differences observed between W303 and SK1 are co-located with the presence or absence of repetitive sequences, such as Ty repetitive elements that could alter origin activity. This also alters the apparent but not real fork velocity, which in turn can give rise to a difference in replication time without any difference in replication origin activity (Figure 34).

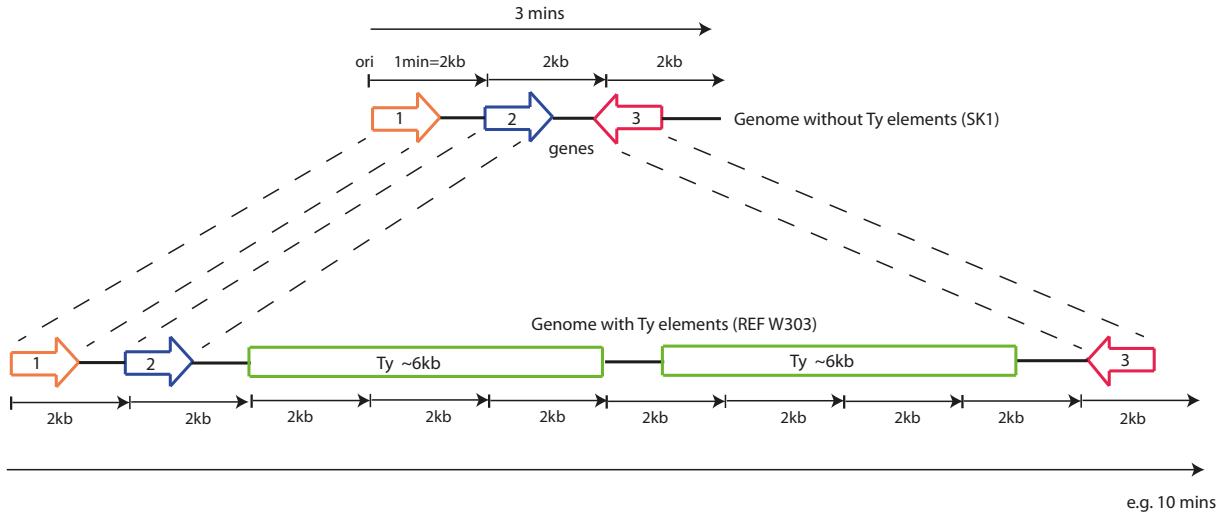


Figure 34: Cartoon showing the presence of Ty elements in between the genes and their role in changing the replication time. 1,2 3 indicates genes

Another difference between the two genomes is found on Chr IV at around 1100kb – 1200kb. Resequencing data (SGRP) from SK1 suggests loss of genomic sequence from the intergene that contains *ARS432*, consistent with the collapse of the *HXT7-HXT6* gene pair (these are genes that result from a local tandem repeat) to give a single *HXT* gene (Sasaki et al., 2013). Therefore it is possible that *ARS432* (and flanking sequence) are lost in SK1 and related strains. This would then result in SK1 having only a single origin in this region and hence a later replication time.

One interesting example is at *ARS1323* where the origin appears to be less active in SK1 than in W303. Resequencing data shows that there is a 4bp insertion with the ORC–binding site in SK1, relative to W303. This might be responsible for delayed replication origin timing (Figure 35).

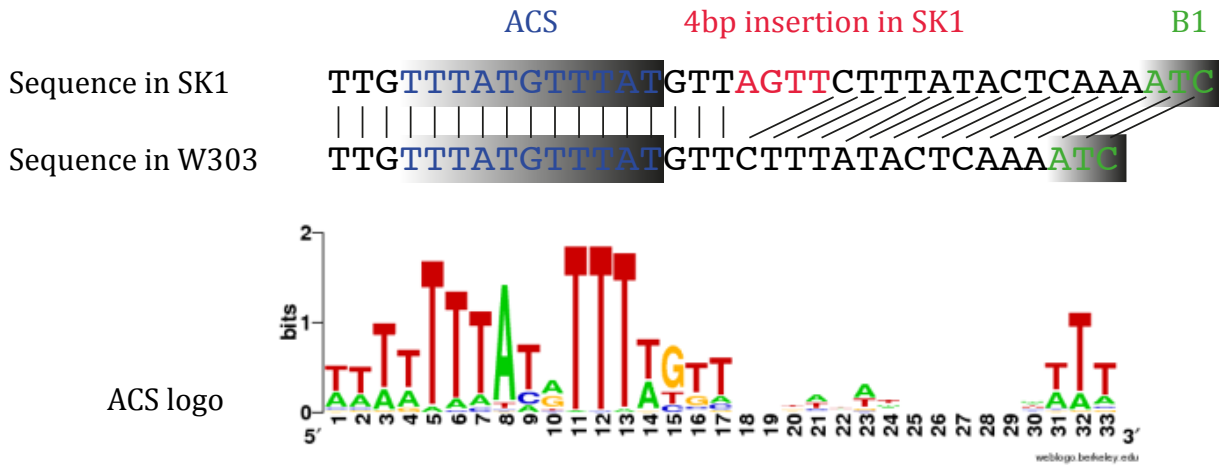


Figure 35: Sequence alignment in SK1 and REF genome showing a difference of 4bp insertion in SK1.

Table 10: List of differences between replication profiles of W303 and SK1 strains

Genomic location	Replication differences	Possible explanations for differences
Chr IV: 850-880kb	SK1 early than W303	2*Ty elements in W303 but not in SK1 (see Fig 33).
Chr IV: 1100-1200kb	SK1 is later than W303	-Two genes (HXT7-HXT6) in W303, but only one HXT gene in SK1. - Likely loss of *ARS432* in SK1.
Chr VII: 550-600kb	SK1 early than W303	Loss of 2 Ty elements in SK1 relative to W303 (Fig 33).
Chr VIII: 100-175kb	Loss of activity in SK1	In W303 strain, *ARS807* is flanked by LTRs and *ARS807* has been lost in SK1.
Chr XI: 250-325kb	SK1 is later than W303	- In W303 strain, *ARS1107.5* is flanked by an LTR and tRNA. - Resequencing (SGRP) data suggests that in SK1 there is a full-length Ty1 adjacent to the origin.
Chr XIII: 175-275kb	SK1 early than W303	Resequencing (SGRP) data suggests that two Ty elements are missing in SK1 (Fig 33).
Chr XIII: 575-675kb	SK1 later than W303	Presence of a predicted ACS for *ARS1323* with a 4 bp insertion in SK1 (SGRP data).
Chr XV: 0-100kb	SK1 early than W303	*ARS1506.5* appears more active in SK1 than W303. Could be related to difference in telomere proximal sequences.
Chr XV: 925-1000kb	'faster' moving leftward forks in SK1	Resequencing (SGRP) data suggests the loss of two Ty elements in SK1 relative to W303 (Fig 33).
Chr XVI: 750-800kb	SK1 early than W303	Resequencing data suggests an insertion close to the origin, which is flanked by LTRs in W303.

3.3.3 Synchronous meiotic time course of SK1

The analysis of yeast meiosis has been greatly facilitated by the ability to generate cultures that are synchronized, enabling a meiotic time-course to be established in which the key molecular events of recombination at the DNA level are set in the context of nuclear and chromosomal changes (Armstrong, 2013). I used these meiosis synchronization approaches with the aim of generating a meiotic DNA replication profile.

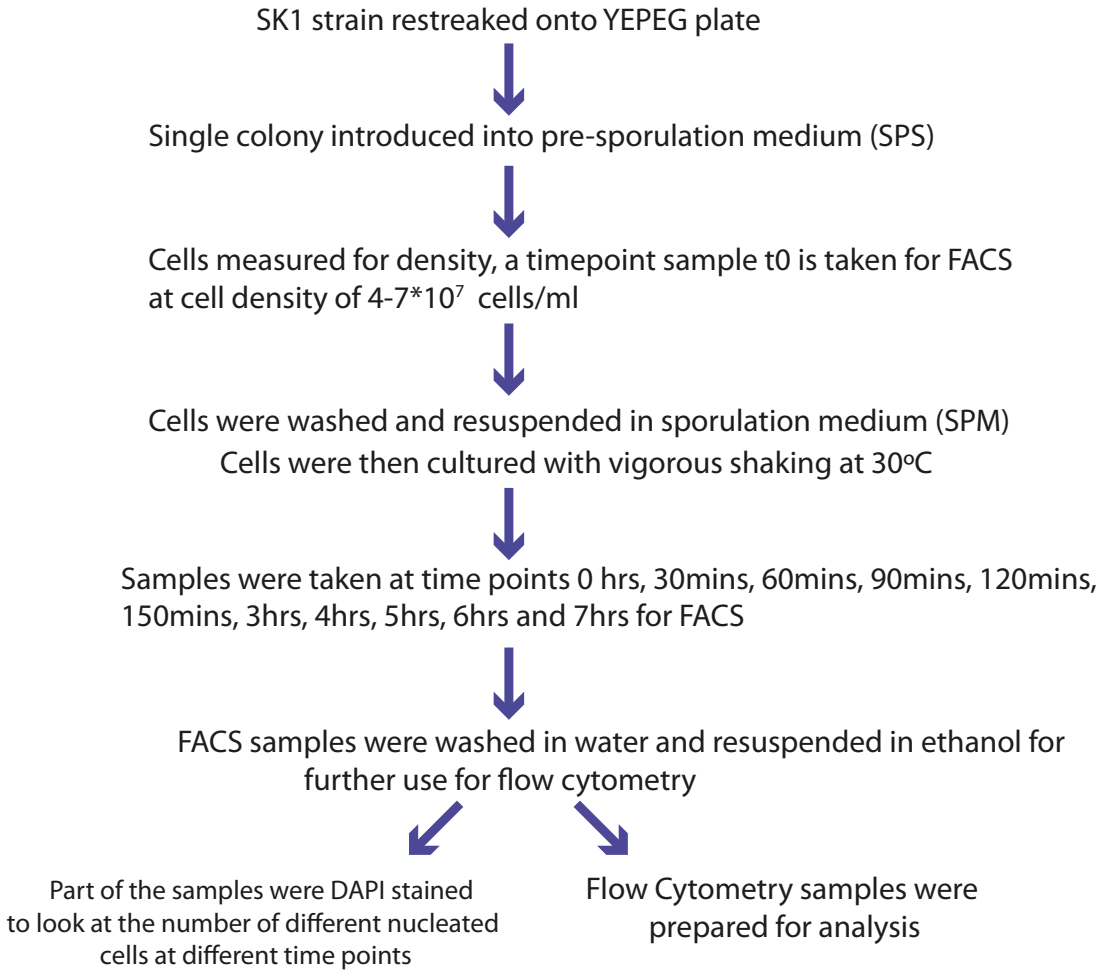


Figure 36: Schematic of meiotic time course experiment.

Following the procedure shown in Figure 36, the cells were allowed to synchronously enter meiosis. Samples were collected at different time points to analyze the progression of cells and events occurring during the course of meiosis. The samples collected were used for analyzing the replication activity during that particular time point and also to look at the physical structure of the cells. At each time point cells were counted for the number of mono, bi, tri and tetra nucleated cells. The cells were stained with DAPI for visualization of number of nuclei in the cells (Figure 37).

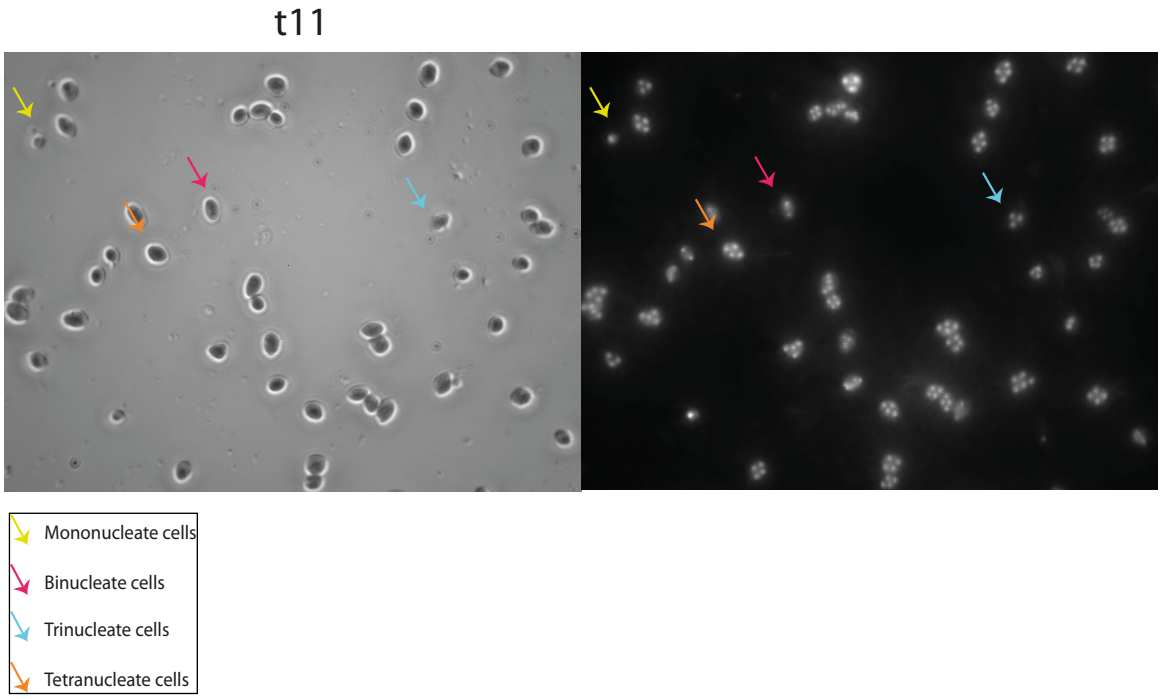


Figure 37: Microscopic image of DAPI stained SK1 cells during meiotic time course experiment showing various nucleated cells. Left panel shows the normal microscopic image of the cells at time point t11, and the right panel shows the image of the DAPI stained cells under a fluorescence microscope.

The DAPI stained cells were visualized under a fluorescence microscope. At each time point, cells were counted and the number of nucleated cells was recorded. This gives an indication of the progression of cells into meiosis. From the data collected, the rise of tetra-nucleated cells and the decline of mono and bi nucleated cells was seen after 6 hours indicating that the cells were completing meiosis II after ~7-8 hours. Spore formation was also quantified. At each of the time points samples were taken and number of spores were counted using a haemocytometer. The production of spores was first observed after 7 hours and then a steady rise in the number of spores produced was observed. Figure 38 shows the percentage of each type of cells and spores at each time point.

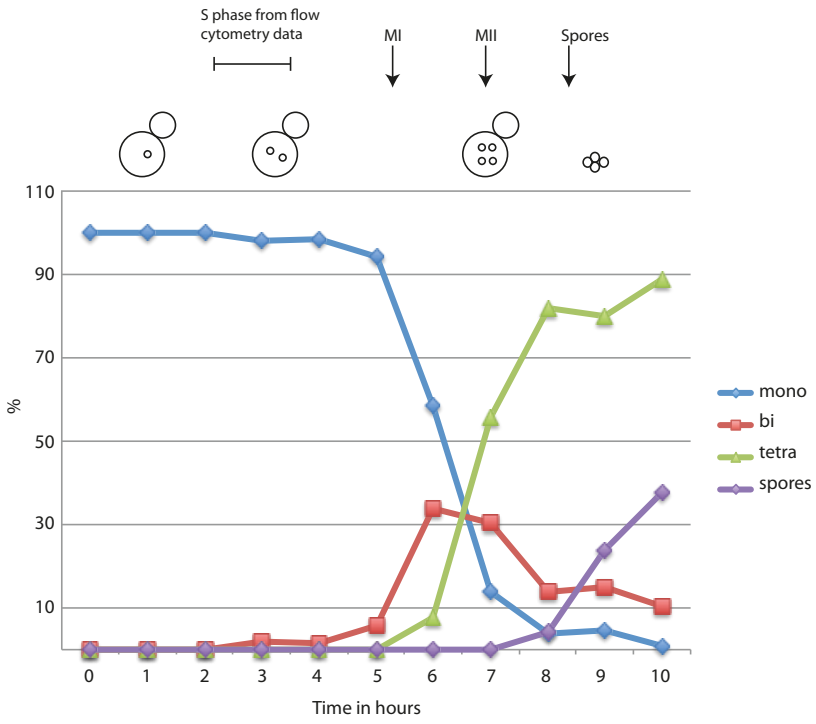


Figure 38: Percentage of mono, bi, and tetra nucleated cells and spores at different time points. X-axis indicates the time in hours at which each of the samples were taken and analyzed. Y-axis indicates the percentage of cells at each of these time points.

DNA replication of SK1 strain was analyzed in the synchronous meiosis using flow cytometry (Figure 39). Flow cytometry was used to assess the degree of synchrony. DNA content was represented in two peaks G1 (n) and G2 (2n) cells. The area between the two peaks represents the S phase cells. Decline of G1 peak and increase in S phase cells started from around ~2.5 hours, indicating that the cells enter into pre-meiotic S phase in around 2.5 hours. Increase in S phase cells was seen in between 150-210 minutes indicating the approximate length of S phase to be around 60 minutes. Increase in 2n peak was seen from ~165 minutes (Figure 39). From the flow cytometry data, time points, which were equally spaced, were selected, which include S phase cells. To map origin locations, replication forks were stalled close to their initiation sites; therefore, copy number enrichment was limited to origins. Genome-wide peak calling was performed to identify regions of DNA synthesis and thus potential replication origin locations. Peak locations allowed the identification of a sequence logo that strongly resembles ACS motifs determined. Therefore, copy number maxima coincide with locations of known replication origins at a resolution sufficient to rediscover the known origin sequence motif. Peak heights represent the proportion of cells that replicated the sequence during the 60 min in HU. If a sequence was replicated in every cell of the population, the copy number would reach a theoretical maximum of 2. A range of peak heights was

observed with a maximum of ~1.8. Differences in the peak height associated with an origin could reflect differences in origin activity. For example, origins might not be licensed in every cell and therefore they would not be competent to activate in some cells. In addition, origins that activate later will experience lower dNTP concentrations, which may result in origin unwinding but no nascent strand synthesis, giving extended regions of single-stranded DNA. Therefore, later-activating origins would be anticipated to give rise to lower peaks as a consequence of reduced DNA synthesis.

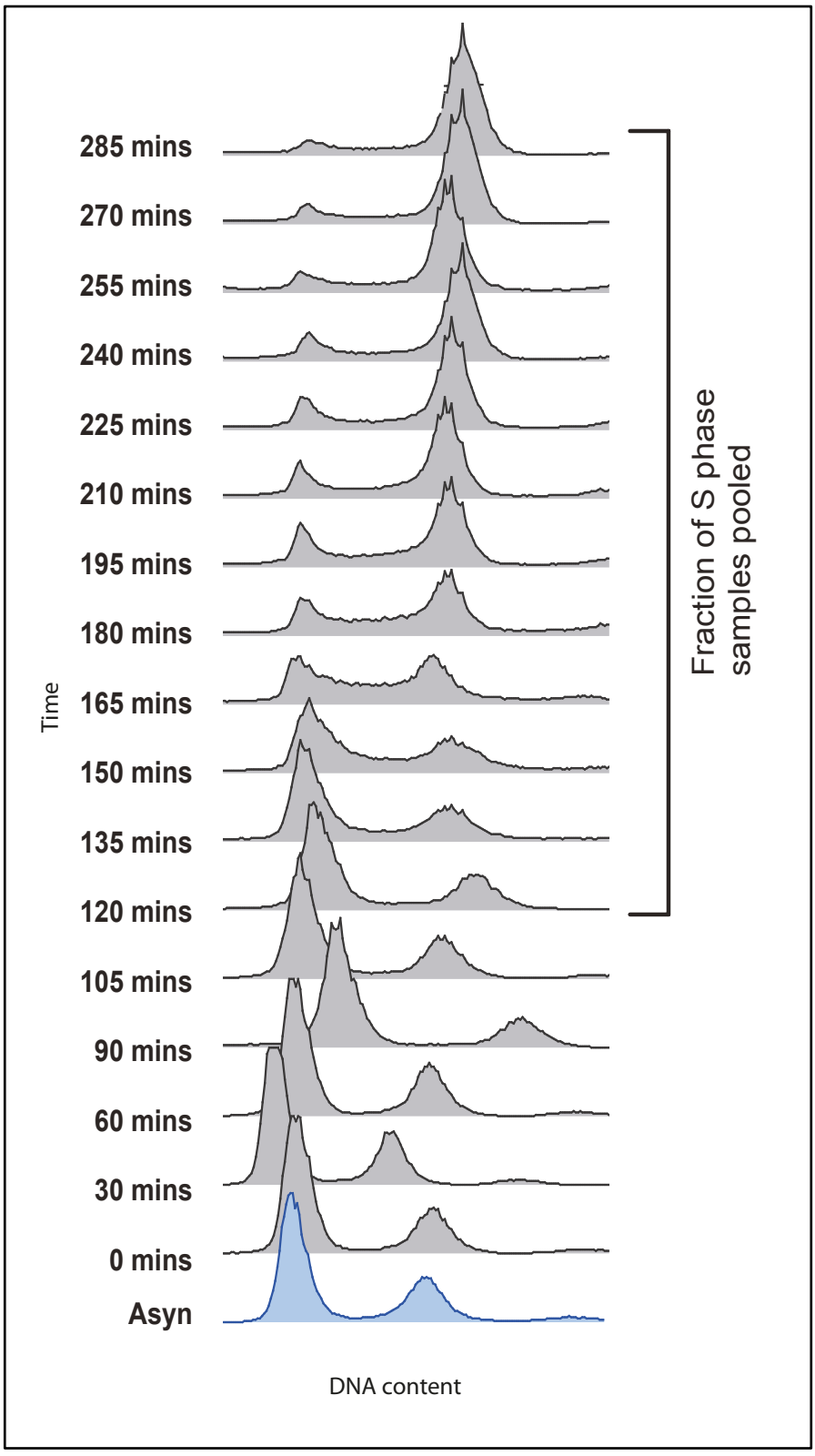


Figure 39: Flow cytometry analyses of synchronous meiotic SK1 cells. Blue coloured area indicates the asynchronous cell culture.

To study the replication dynamics of S phase cells, rather than investigating specific time points, cells were pooled from within a time window of 120 minutes to 285 minutes to enrich S phase cells. The pooled sample is enriched for pre-meiotic S phase cells, but will also contain a large number of pre- and post-S phase cells. Fluorescently activated cell sorting (FACS) was used to enrich the pre-meiotic S phase cells. Figure 40 shows the flow cytometry profile of the cells prior to FACS. In S-phase, copy number of the sequence directly demonstrates the replication timing of the cell. DNA was extracted from the S phase sorted cells. The relative copy number at each genomic location was determined by deep sequencing and normalized to a baseline of 1.0, which resulted in observed maximum peak heights of <2.0.

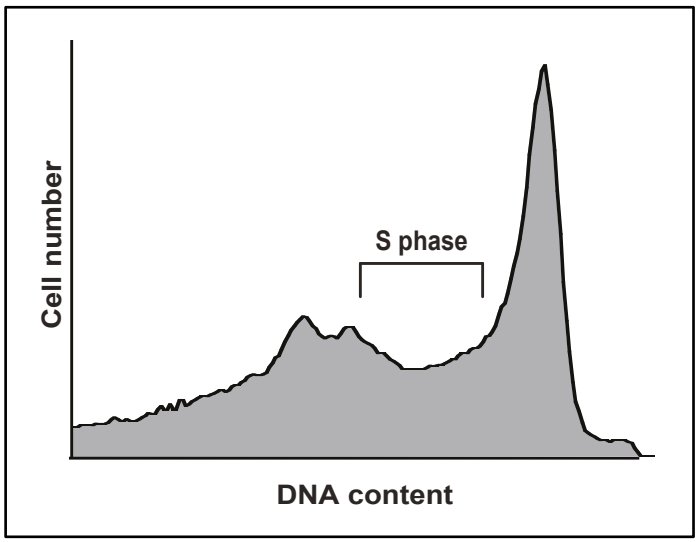


Figure 40: Flow cytometry profile of meiotic-S phase cells sorted by FACS. The S phase marked region indicates the fraction of cells in S phase and the peak indicates the G2 enriched cells.

3.3.4 Generating a meiotic replication profile for SK1

Pre-meiotic genome replication dynamics were assayed using deep sequencing, to measure the relative copy number of replicating cells relative to non-replicating (stationary phase) cells. The replicating sample corresponded to the sorted S phase sample, which was subjected to deep sequencing to determine the relative copy number of each genome location. Figure 41 shows the genome wide replication profile data of pre-meiotic S phase cells. The blue lines in the profiles represent the centromere, which is one of the first genomic locations to replicate during a mitotic cell cycle.

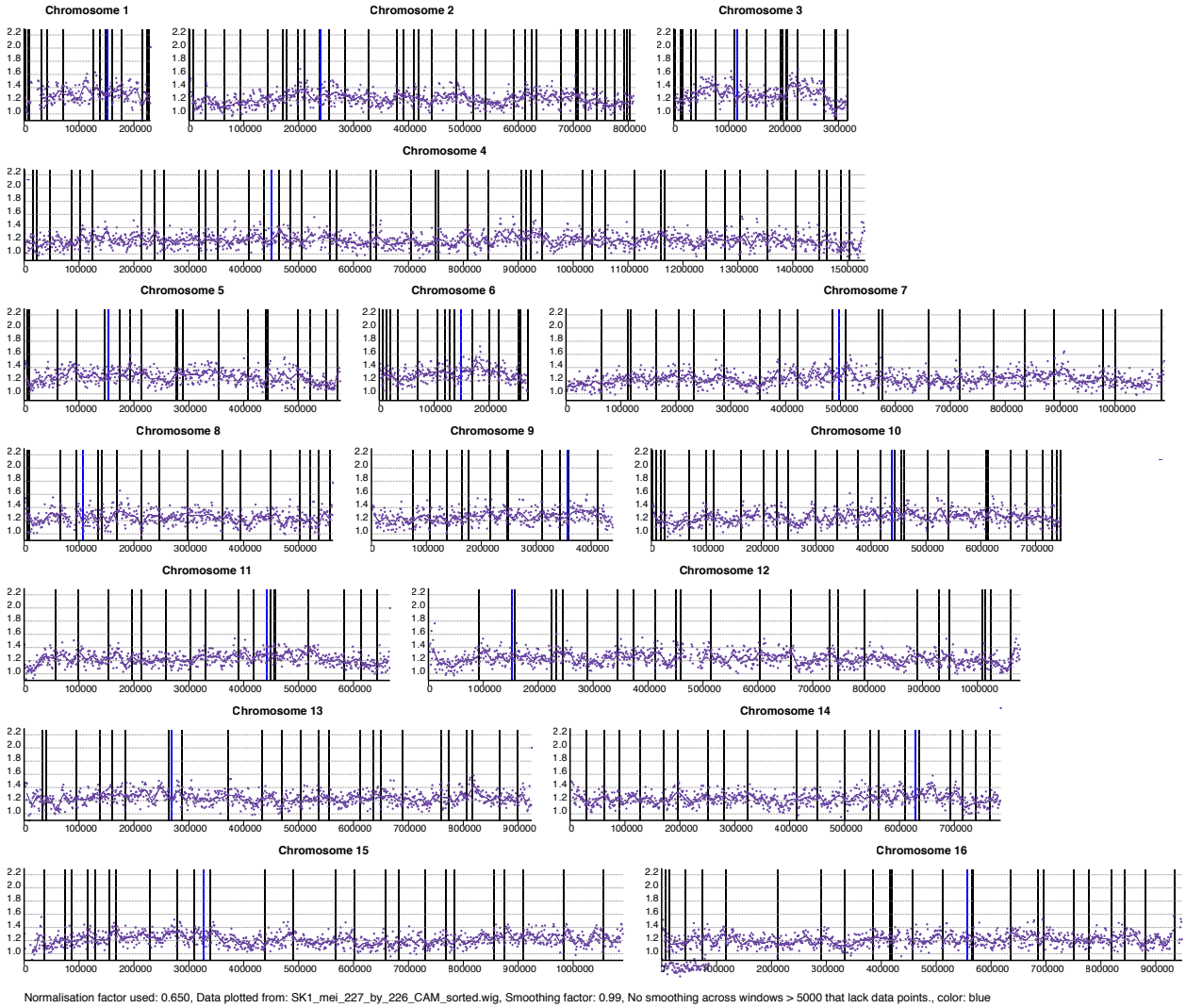


Figure 41: Genome wide meiotic replication profile of SK1. Genome wide replication data of SK1 strain during meiosis. x-axis represents the chromosome coordinates in bp and y-axis represents the relative copy number. The blue vertical line indicates the location of the centromere on the chromosome. Black vertical lines indicate the location of confirmed replication origins.

3.4 Summary

The genome wide replication data from sort-seq of synchronous meiotic time course, did not give the anticipated data. This might be because meiotic DNA replication could be very much more stochastic than the mitotic replication, therefore giving much flatter profiles. Another possible explanation could be a technical problem, such as not representative DNA due to FACS problem or DNA extraction problems from meiotic cells. Other lab members have found that some methods to extract DNA give a flatter profile, indicating that the method of extraction of DNA is very important

to get high quality replication profiles. Although I used a standard approach for DNA extraction, that was successful for the mitotic profile, it is possible that this protocol gave biased DNA recovery from meiotic cells. Other possibility might be the meiotic cells, which give erroneous fluorescence in FACS that gives contamination of non-replicating cells in FACS enriched cells. Due to these limitations, an alternative approach was required for studying the meiotic origin activity.

4 Trancriptionally regulated yeast replication origins

4.1 Introduction

The sites in the genome where the process of DNA replication initiates are called the origins of replication. The process of DNA replication from an origin is bidirectional. Replication origins in yeast are called the Autonomously Replicating Sequences (ARS). The DNA sequence of each of the ARS varies, however they have certain characteristics in common such as rich AT content. Among the eukaryotes, budding yeast *Saccharomyces cerevisiae* replication origins are very well characterized. Several studies have identified the activity and location of replication origins by complimentary microarray-based approaches. These include a dense isotope transfer method, copy number change measurements and mapping pre-RC protein binding sites (Raghuraman et al., 2001) (Wyrick et al., 2001) (Yabuki et al., 2002). Comparative genomics was also used to determine the genome wide location replication origins at a resolution sufficient to identify the sequence elements bound by replication proteins (Nieduszynski et al., 2006b). A list of all the identified origins was compiled in the OriDB database (Siow et al., 2012). Origins are categorized as 'Confirmed', 'Likely' or 'Dubious' depending on the degree of supporting experimental evidence. Various studies have identified and experimentally confirmed 410 replication origins, 203 likely origins and 216 dubious origins (Figure 42).

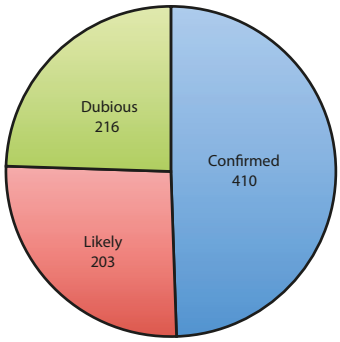


Figure 42: Number and status of replication origins in *S. cerevisiae* in OriDB Numbers were taken from cerevisiae.oridb.org (Jan 2015)

'Confirmed' origins are those, which has been successfully cloned, and origin activity confirmed by ARS assays or chromosomal activity detected by 2-D gel electrophoresis. 'Likely' origins are those where the origin predictions from two or more studies overlap. Origin sites proposed by a single study are grouped as 'dubious' origins. These may include a high proportion of false positives (Muller and Nieduszynski, 2012). Most of the origins confirmed in yeast are present in between the genes, 'Intergenic Origins'. Earlier studies have identified no active origins coinciding with active transcription and the identification of intergenic origin sequences for majority of active origins, implies the possibility of very few origins within the transcription units (Nieduszynski et al., 2006a, Legouras et al., 2006) and that transcription is generally detrimental to origin function (Nieduszynski et al., 2005). Work presented in this chapter aims to characterize replication origins within protein coding genes. Initially genome-wide replication data were thoroughly studied and the location of possible origin sites was compiled. The data confirming origin activity was performed by ARS assays.

4.2 Identification of a set of origins lying within genes

Most of the studies on replication origins in budding yeast to date have identified the presence of replication origins sites between genes. As discussed earlier, it is thought that transcription is detrimental to origin activity; hence most of the origins identified are intergenic origins. One study has identified the presence of origin sequences within protein coding genes (Shirahige et al., 1993). *ARS604* and *ARS605* were confirmed to be present on genes *BLM10* and *MSH4*. Origins were confirmed by ARS assays. Sequences essential for ARS activity were determined for the ARS fragments by deletions and mutations. I set out to determine whether there are additional yeast origins within protein coding genes.

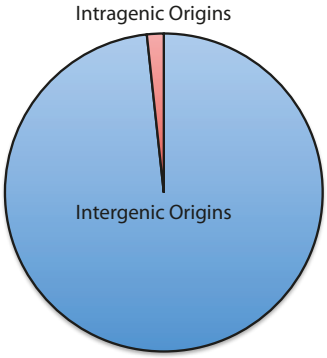


Figure 43: Pie chart showing the number of origins lying between gens (Intergenic) and within genes (Intragenic). Of the total number of confirmed origins in yeast (410), 403 origins are intergenic origins and 7 origins were found to be intragenic origins.

4.2.1 Analysis of independent candidate datasets (mostly published)

Chromatin immunoprecipitation (ChIP) of origin binding factors (ORC [origin recognition complex] and Mcm2-7) facilitated identification of replication origin regions in *S. cerevisiae* (Wyrick et al., 2001). Careful analysis of genome-wide replication profiles and ChIP data was done with the help of a colleague in the lab Carolin A Müller. Data from genome-wide analysis of mitotic replication profile has identified a set of origins, which may lie within a gene (Table 11).

Table 11: Replication origins in yeast, those lie within genes/ORFs.

Chromosome	Gene	Origin	OriDB status
I	*YAL067C*	*ARS103*	Confirmed
VII	*YGL145W*	*ARS711*	Confirmed (Chromosomally inefficient)
VII	*YHR018C*	*ARS808*	Confirmed (Chromosomally inefficient)
IX	*SPO22*	*ARS-SPO22*	Confirmed in this study
IX	*HOP1*	-	Clear Mcm4 ChiP peak observed, indicating presence of origin
X	*YJL095W*	*ARS1009.5*	Confirmed (Chromosomally inefficient)
X	*YJR036C/ HUL4*	*ARS1017.5*	Confirmed (Chromosomally inefficient)
XII	*SPO75*	*ARS1207*	OriDB Dubious ARS. Confirmed in this study
VIII	*YHR054C*	*ARS810*	Confirmed (Chromosomally inefficient)
X	*YJR159W (SOR1)*	*ARS1024*	Confirmed (Chromosomally inefficient)
XI	*YKL027W*	*ARS1112*	Confirmed (Chromosomally inefficient)
XI	*YKR009C (FOX2)*	*ARS1114.5*	Confirmed (Chromosomally inefficient)
XI	*YKR091W (SRL3)*	*ARS1120*	Confirmed (Chromosomally efficient)
XIV	*YNL303W*	*ARS1406*	OriDB Dubious ARS

VI	*MSH4/ YFL003C*	*ARS605*	Confirmed (Chromosomally efficient)
II	*YSW1/ YBR148W*	*ARS218*	Confirmed (Chromosomally efficient)
IV	*SPR28/ YDR218C*	*ARS427.5*	Confirmed (Chromosomally inefficient)
IV	*ZIP1/ YDR285W*	*ARS430.5*	Confirmed (Chromosomally efficient)
II	*YBR285W*	*ARS227*	Confirmed (Chromosomally efficient)
V	*YEL023C*	*ARS509*	OriDB likely ARS (Chromosomally inefficient)
VI	*YFL007W /BLM10*	*ARS604*	Confirmed (Chromosomally inefficient)

After careful identification of genome-wide replication data, a set of origins within both meiosis specific and non-meiosis specific genes were identified. In addition to the set of origins identified in Table 11, there is a set of origins, which were taken from previously published data and which are present on genes. These identified origins were chosen and were thoroughly studied. ARS assays were used to confirm the location of origins present in a larger and smaller fragment of the gene. Nine origins were identified to be present within genes (Table 12). Interestingly, all the origins confirmed to be present on genes, have meiotic function, except for one origin (*ARS604*). Of the six origins, which were found to have meiotic function, five of them (*ARS605*, *ARS218, ARS427.5, ARS430.5* and *ARS227*) were confirmed origins and *proARS509* was likely to be a confirmed region for ARS activity. Previously confirmed intragenic origins, which have specific meiotic function were selected and further studied to find out the origin activity, relation between activity of origin and the transcription of gene.

4.2.2 ARS assays for confirmation of origin location

Likely and unconfirmed origins were analyzed for origin activity using ARS assays. Conventional plasmid based ARS assays were used which required cloning of potential replication origin site, thereby restricting the size of origin fragment. Different fragment sizes were tested to find out the precise location of origin activity. The Saccharomyces Genome Resequencing Project (SGRP) generated whole genome libraries for *S.cerevisiae* strains.

SGRP clones contain an average 3-5 kb of chromosomal DNA inserted into the *lacZ* gene of a standard cloning vector, making these clones suitable for recombination-based ARS assays (Figure 44) (Nieduszynski and Donaldson, 2009). Origin activity was confirmed through ARS assay. Therefore, SGRP clones provide a powerful tool for rapid screening of ARS activity.

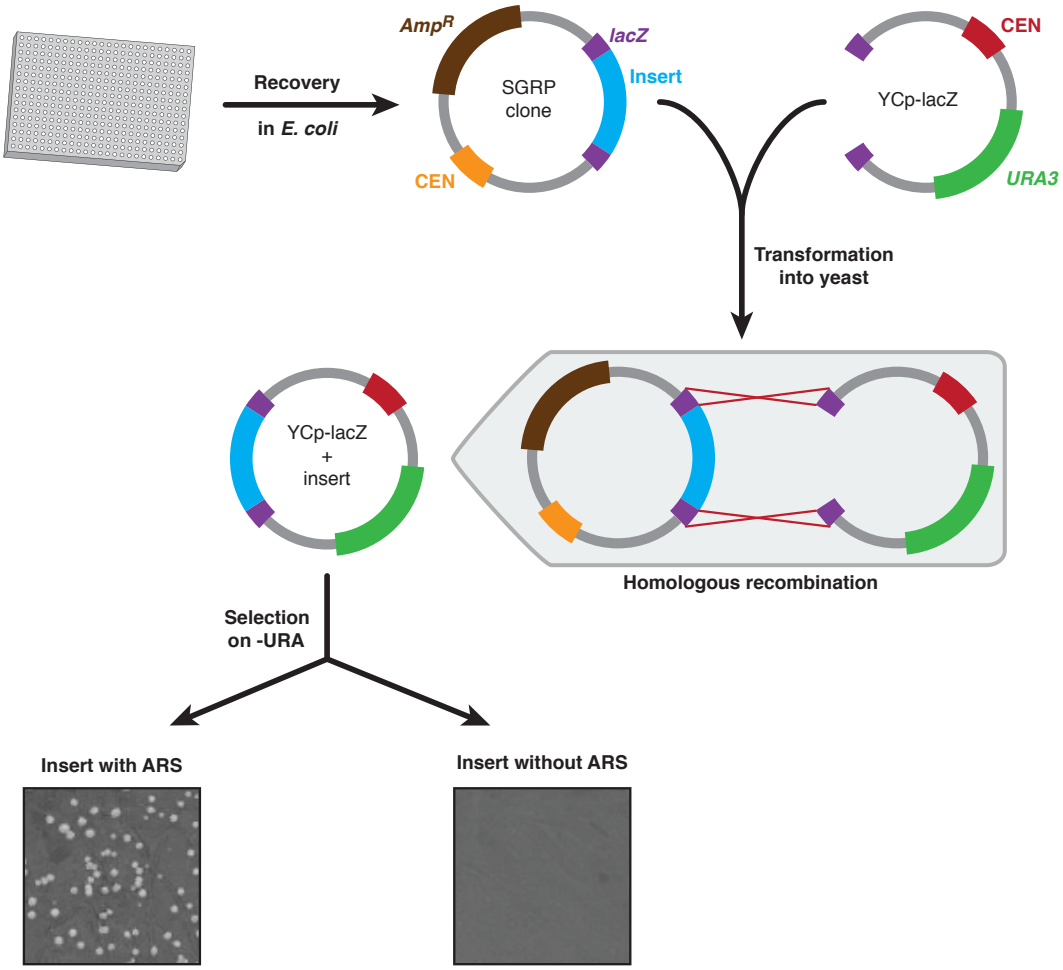


Figure 44: Strategy of recombination-based ARS assays using SGRP clones. Yeast cells lacking the *URA3* gene are co-transformed with an SGRP clone and linearized YCp-lacZ vector. A plasmid that contains the SGRP clone insert in the YCp-lacZ backbone will form through homologous recombination. The plasmid is required for colony growth on media lacking uracil. In turn, plasmid maintenance depends on the presence of an ARS within the DNA insert from the SGRP clone. (Figure adapted from thesis of Carolin A Müller, 2012)

Recombinant ARS assay were performed for proposed origin regions. Proposed origin regions were identified and SGRP clones covering the area were selected for ARS assays. ARS activity was detected by the presence of colonies. SGRP clones covering the region and the yeast cells lacking the *URA3* gene were co-transformed with SGRP clone and linearized YCp-lacZ vector. Plasmid maintenance depends on the presence of an ARS within

the DNA insert from the SGRP clone. ARS function was assayed for all the selected origins and confirmed for origin activity. SGRP clones for sites of predicted origins within genes *SPO75* and *SPO22* were identified and selected for recombinant ARS assay.

Spo75 is a meiosis-specific protein of unknown function. *SPO75* is required for spore wall formation during sporulation, however non-essential for both nuclear divisions during meiosis (Coluccio et al., 2004). The gene ontology overview of *SPO75* gene if it is required for ascospore formation and ascospore wall assembly. SGRP clones covering the *SPO75* gene (SK1-11i19, SK1-72m09) were identified from the Sanger genome browser (http://gbrowse.sanger.ac.uk). After identifying the SGRP clones, various sets of primers were designed to clone different regions of the gene, to test for the existence of origin activity in different fragments of the gene. Fragments were amplified with the different sets of primers by PCR and confirmed by gel electrophoresis. The PCR fragments were cloned into pGEM-Teasy vector that in turn were co-transformed with linearized YCp-lacZ vector into yeast. Resulting homologous recombination generates plasmid required for colony growth on media lacking uracil. On selective media only a plasmid with a functional ARS give colonies. Therefore colony growth depends on the presence of an ARS within the DNA fragment that was amplified by PCR (Figure 45).

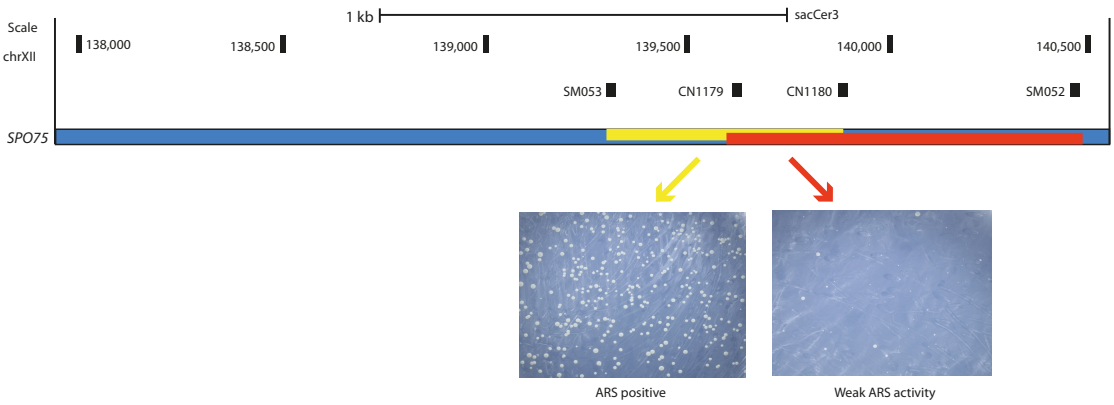


Figure 45: ARS assay of *SPO75* gene. Cartoon showing *SPO75* gene and the fragments tested for ARS activity. SGRP clones covering the *SPO75* gene were identified and additional fragments were cloned with the set of primers indicated in the cartoon. The fragments were cloned and tested for ARS activity. The plasmid carrying the fragment with ARS activity gives colonies when plated on selective media. The plasmid pSM099, cloned with primers SM053 and CN1180, was confirmed ARS positive by the presence of colonies on a selective media. The other plasmid pSM098 cloned with primers CN1197 and SM052 showed very light and small number of colonies developed after longer incubation time. This weak ARS activity of the fragment might be due to the overlapping fragment with confirmed ARS activity. This ARS assay gives us a clear confirmation of the presence of origin on *SPO75* gene.

Spo22 is also a meiosis-specific protein, which is essential for chromosome synapsis and is involved in completion of nuclear division during meiosis and induced during early meiosis (Coluccio et al., 2004) (Tsubouchi et al., 2006). SGRP clones covering the *SPO22* gene (SK1-8p14, SK1-24e20) were identified from the Sanger genome browser (http://gbrowse.sanger.ac.uk). After identifying the SGRP clones, various sets of primers were designed to clone different regions of the gene, to test for the existence of origin activity in different fragments of the gene. Fragments were amplified with different sets of primers by PCR and confirmed by gel electrophoresis. The PCR fragments were cloned into pGEM-T easy vector that in turn were co-transformed with linearized YCp-lacZ vector in to yeast. Resulting homologous recombination generates plasmids that are required for colony growth on media lacking uracil. On selective media only plasmids with a functional ARS give colonies. Therefore colony growth depends on the presence of an ARS within the DNA insert from the SGRP clone (Figure 46).

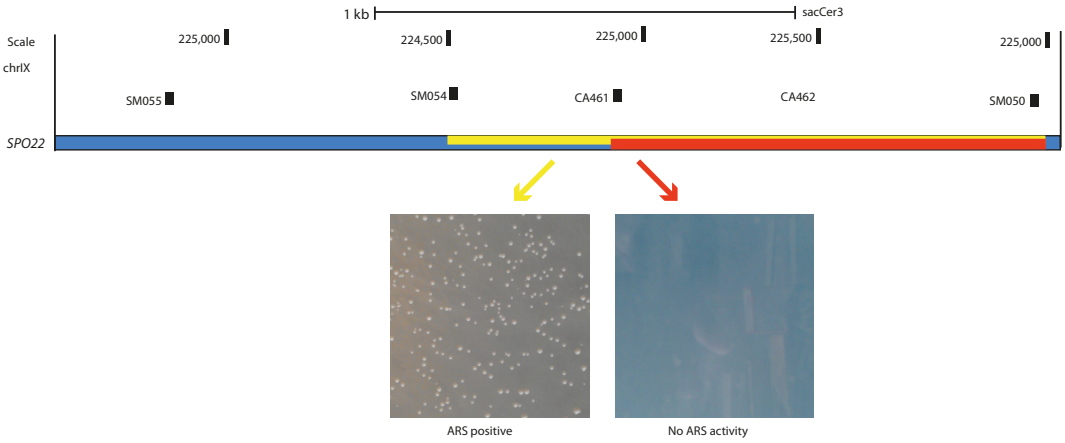


Figure 46: ARS assay of *SPO22* gene. Cartoon showing *SPO22* gene and the fragments tested for ARS activity. SGRP clones covering the *SPO22* gene were identified and cloned with the set of primers indicated in the cartoon. The fragments were cloned and tested for ARS activity. The plasmid carrying the fragment with ARS activity gives colonies when plated on selective media. The plasmid pSM088, cloned with primers SM054 and SM050, was confirmed ARS positive by the presence of colonies on a selective media. The other plasmid pSM084 cloned with primers CA461 and SM050 showed no colonies, indicating the absence of ARS activity

Yel023c is a putative protein of unknown function. *YEL023C* is a non-essential gene whose expression is increased greatly during sporulation (Chu et al., 1998). SGRP clones covering the *YEL023C* gene (SK1-38l24) were identified from the Sanger genome browser (http://gbrowse.sanger.ac.uk). After identifying the SGRP clones, various sets of primers were designed to clone different regions of the gene, to test for the existence

of origin activity in different fragments of the gene. Fragments were amplified with different sets of primers by PCR and confirmed by gel electrophoresis. The PCR fragments were cloned into pGEM-Teasy vector that in turn were co-transformed with linearized YCp-lacZ vector in to yeast. Resulting homologous recombination generates plasmid is required for colony growth on media lacking uracil. On selective media only plasmid with a functional ARS give colonies. Therefore colony growth depends on the presence of an ARS within the DNA insert from the SGRP clone (Figure 47).

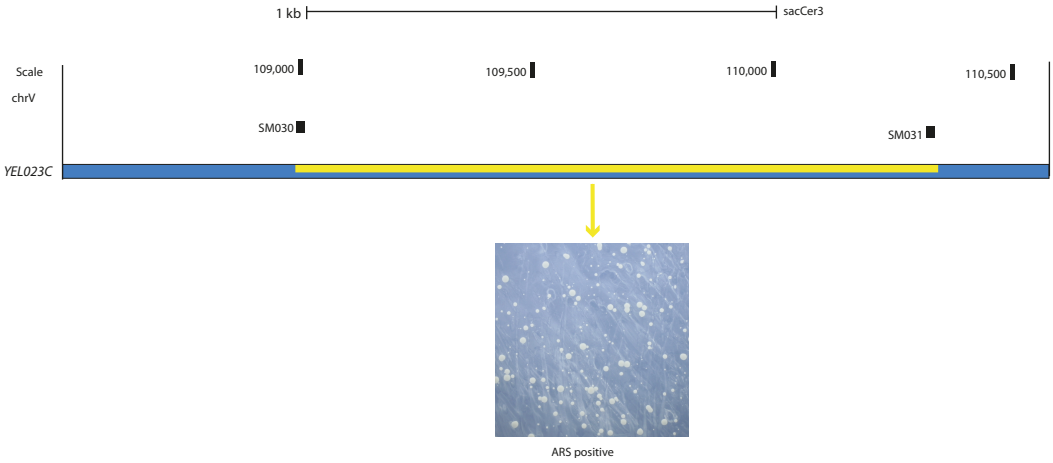


Figure 47: ARS assay of *YEL023* gene. Cartoon showing *YEL023* gene and the fragments tested for ARS activity. SGRP clones covering the *YEL023* gene was identified and cloned with the set of primers indicated in the cartoon. The fragments were cloned and tested for ARS activity. The plasmid carrying the fragment with ARS activity gives colonies when plated on selective media. The plasmid pSM063, cloned with primers SM030 and SM031, was confirmed ARS positive by the presence of colonies on a selective media indicating the presence of ARS activity

ARS assay thus provide a high throughput assay for testing origin activity. ARS assay can test origin activity for fragments ranging from nearly 200 bp sequences ranging up to ~25 kb.

4.3 Analysis of genes containing origins

4.3.1 Gene annotations

Having found a set of origins that lie on genes, I then aimed to determine whether the genes containing origins have any common features. I initially looked at GO term analysis. The GO (gene ontology) terms used for describing the genes were related to meiotic function. Earlier studies have identified an origin *ARS605*, which is present on the gene *MSH4*. It is involved in meiotic recombination and for maintaining normal levels of crossing over. In the absence of *MSH4*, crossing over is reduced by two- to three fold. Msh4 localizes to sites of synapsis initiation and promotes SC formation. Msh4 colocalizes with Zip2 to discrete foci on meiotic chromosomes, has homology to bacterial MutS protein (Novak et al., 2001).

Ysw1 is a spore-specific protein required for normal prospore membrane formation; interacts with Gip1, which is the meiosis-specific regulatory subunit of the Glc7 protein phosphatase; expressed specifically in spores and localizes to the prospore membrane. Sporulation is partially defective in *ysw1*Δ mutant, and cytological analysis revealed that septin structures are perturbed and prospore membrane extension is aberrant in *ysw1Δ* cells (Ishihara et al., 2009). The GO terms proposed for *YSW1* are ascospore-type prospore membrane assembly, septin complex, molecular function and membrane assembly. *SPR28* is a sporulation-specific homolog of the CDC3/10/11/12 family of genes; meiotic septin expressed at high levels during meiotic divisions and ascospore formation. The common GO terms associated with *SPR28* are meiotic spindle, septin complex, sexual sporulation, which indicates that the *SPR28* gene plays a role during meiosis.

Zip1 is a transverse filament protein, which is required for normal levels of meiotic recombination (Shinohara and Shinohara, 2013). GO terms include meiotic cell cycle, synapsis, reciprocal meiotic recombination etc. Ybr285w is another putative protein with unknown function. *YBR285W* is a non-essential gene (Duenas et al., 1999). No specific GO terms were described because of the unknown function of the gene. *BLM10* is the only mitotic gene on which an origin is present. No known meiotic function is associated with the gene.

Considering the origins that were identified to be present on genes, a high proportion of them are present on meiotically active genes. Each of the origins identified and the genes on which they are present were thoroughly studied (Table 12). Given significant enrichment

for GO terms relating to meiosis, I then looked at the meiotic gene expression data for all genes that contained an origin of replication.

Table 12: List of gene on which origins are present and their GO terms

Chromosome	Gene	Origin	Meiotic expression	**GO terms**	**Function of gene**
VI	*MSH4/ YFL003C*	*ARS605*	Early expression	Mismatched DNA binding, Meiotic cell cycle, DNA binding	Meiotic recombination, required for normal crossing over, bacterial MutS homolog
II	*YSW1/ YBR148W*	*ARS218*	During meiosis I	Septin complex Molecular function Membrane assembly	Required for normal prospore formation
IV	*SPR28/ YDR218C*	*ARS427.5*	During meiosis I	Septin complex Meiotic spindle Sexual sporulation	Sporulation specific homolog of CDC3/10/11/12 family of genes, expressed at high levels during meiotic divisions
IV	*ZIP1/ YDR285W*	*ARS430.5*	Early expression	Synapsis Meiotic cell cycle Reciprocal meiotic recombination	Transverse filament protein of synaptonemal complex, required for meiotic recombination
II	*YBR285W*	*ARS227*	Early expression	-	Putative protein of unknown function, not an essential gene
V	*YEL023C*	*ARS509*	During meiosis I	-	Putative protein of unknown function, not an essential gene, expression is increased greatly during sporulation
VI	*YFL007W/ BLM10*	*ARS604*	Low expression	-	-
IX	*SPO22*	*ARS-SPO22*	Early expression	Regulation of synaptonemal complex assembly.	Meiosis-specific protein, involved in completion of nuclear divisions during meiosis
XII	*SPO75*	*ARS1207*	During meiosis I	Ascospore formation Ascospore wall assembly	Required for spore wall formation, meiosis specific protein

4.3.2 Gene expression data

Having found that the genes on which ORFs are present have an active function during meiosis, I then looked at the expression of these genes during meiosis. Data for meiotic gene expression was taken from an RNA sequencing experiment where the experiment was optimized for meiotic synchrony and dense time points were oversampled for meiotic transitions (Brar et al., 2012). The data for selected gene expression was plotted (Figure 48). The ORF containing genes, which were found to have meiotic functionality, were also seen to be expressed during meiosis. However, the expression varied for each of the

genes. Data suggests the expression of various genes at different stages of meiosis. Some of the genes (*MSH4, ZIP1, YBR285W*) are expressed early during pre-meiotic S phase and some are expressed later during meiosis (*SPR28, YEL023C, YSW1*). The time between 0-2 hours is the pre-meiotic S phase when DNA replication takes place. Gene expression is calculated from the reads per kilo base per million reads (RPKM) at each time point (Figure 48). Since, each of the genes have a varied expression during meiosis, I then looked at the mitotic expression of the genes to see whether the genes are expressed during mitosis.

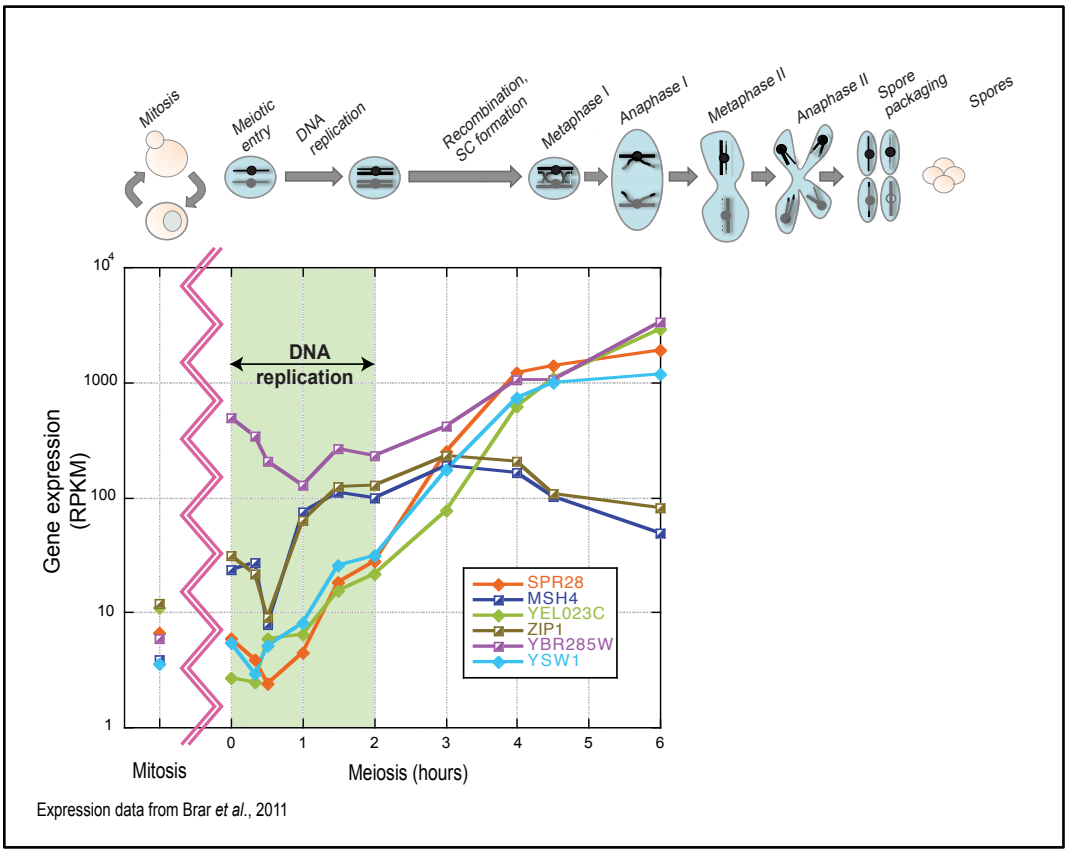


Figure 48: Meiotic expression of selected genes from an RNA seq experiment (Brar *et al* 2012.,)

Data was adapted from the same experiment to study the mitotic expression of the genes. Mitotic expression was analyzed for the thousands of genes in the same study and also for the origin containing genes. Data was analyzed in such a way that the percentage of the total genes falling in a specific expression bin was calculated and compared against the origin containing genes (Figure 49). After careful analysis, it was found that the mitotic expression of origin containing genes was lower when compared to the total number of genes. This indicated that the origin containing genes, which have an active expression during pre-meiotic S phase, have lower expression during mitosis.

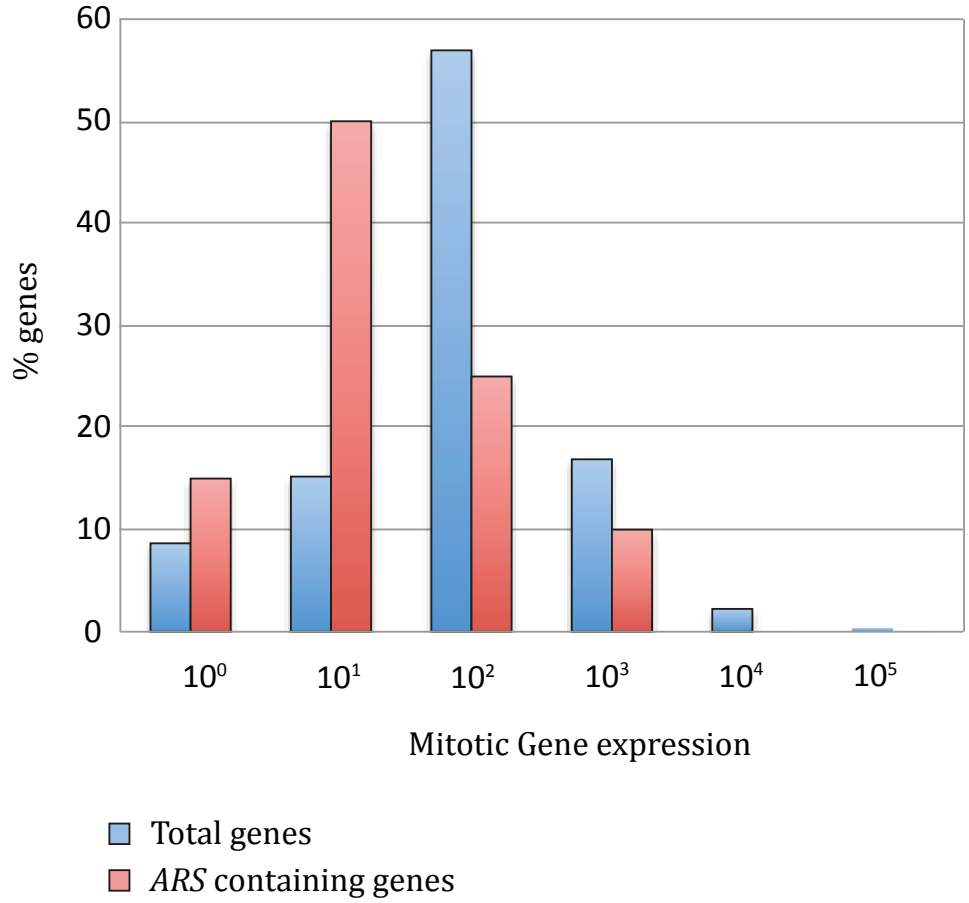


Figure 49: Mitotic expression of all genes and *ARS* containing genes from an RNA seq experiment (Brar *et al.,*)

Having analyzed the mitotic and meiotic expression of the genes, I went on to study the activity of origins present on these genes. My main aim was to find out if there is any relation between the gene expression and origin activity.

4.4 Meiotic plasmid loss assay

We have designed a plasmid loss assay for determining the meiotic activity of the origins. The fragments of origins of interest were cloned and the resulting plasmids were assayed to find out the activity of particular origin fragment (See Materials and Methods). The constructed plasmids were transformed into SK1 strain and were allowed to grow on selective medium (-ura). The strain with the plasmid was allowed to sporulate on the sporulation medium and tetrads were dissected. The spores were allowed to grow on complete

media (YEPD). The spores were then transferred on to selective media to study the plasmid inheritance via spore viability (Figure 50). Different selective medias were selected depending on the presence of various auxotrophic markers on the chromosome. Previously 2D gels have been used to find out the activity of replication origins during meiosis. Given the complexity of 2D gel experiment, I have designed a simpler way to find out the activity of an origin on a plasmid during meiosis.

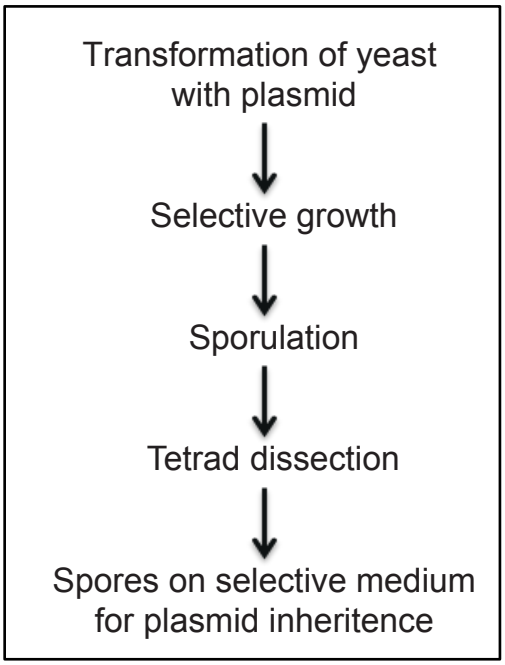


Figure 50: Schematic of Meiotic loss assay

4.4.1 Initial assay developed

Initially, at the beginning of the study a simpler assay was developed to look at the plasmid inheritance via spore viability. A schematic of how the plasmid inheritance was studied is represented in figure 51. There are three instances to be considered while studying plasmid inheritance. The first instance is where the plasmid is lost prior to the cell entering meiosis, i.e. the plasmid is lost during mitosis. In that case, the plasmid never enters the meiotic cycle and hence none of the spores inherit the plasmid. When the plasmid is not inherited, the germinated spore cannot grow on selective media (-ura). In figure 51, the top instance explains the case where the plasmid was previously lost during mitosis. The second instance is where the cell has a single plasmid after the transformation event. If the plasmid replicates successfully during pre-meiotic S phase, segregation of the two daughter plasmids occur during meiosis II and the end of the meiosis events, two out of four daughter spores

inherit the plasmid and remain viable on a selective media. So, at the end of the meiotic events, if a tetrad has two viable spores, this confirms plasmid origin activity. Presence of only one spore at the end of meiotic events indicates plasmid origin inactivity. The mating type of each spore was confirmed on –trp media.

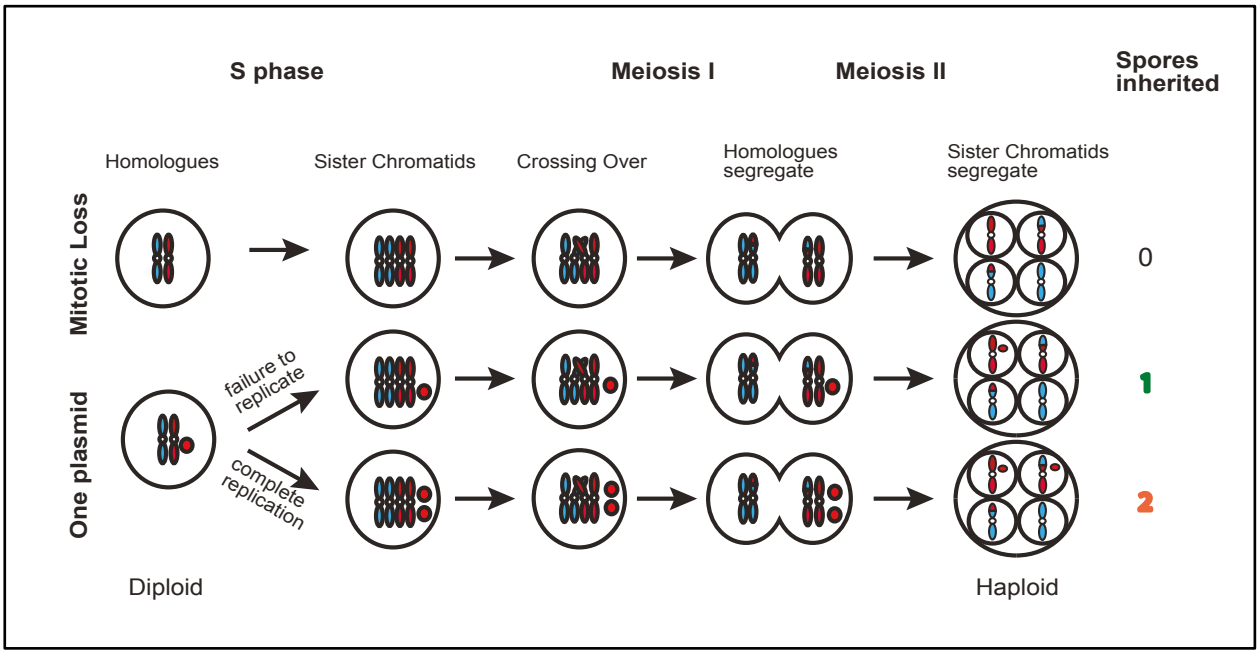


Figure 51: Schematic of various possibilities of plasmid loss during meiosis. The schematic explains the two strategies where the cell enters the meiotic division, i) cell carrying no plasmid and ii) cell carrying a plasmid

4.4.2 Improved assay

With the initial assay developed, the replication origin activity was assayed, however the differences in meiosis I and meiosis II events were not clearly identified. To identify the events taking place during meiosis I and meiosis II, we further proposed an extended schematic, which can explain the difference (Figure 52). In the initial assay developed, only the cells carrying a single plasmid were tested for origin activity. The "improved" assay allows the analysis of cells that starts with two plasmids. After the transformation of a plasmid with yeast cells, there is a chance of a cell acquiring no plasmid, single plasmid or two plasmids. Cell acquiring no plasmid is a case of mitotic loss.

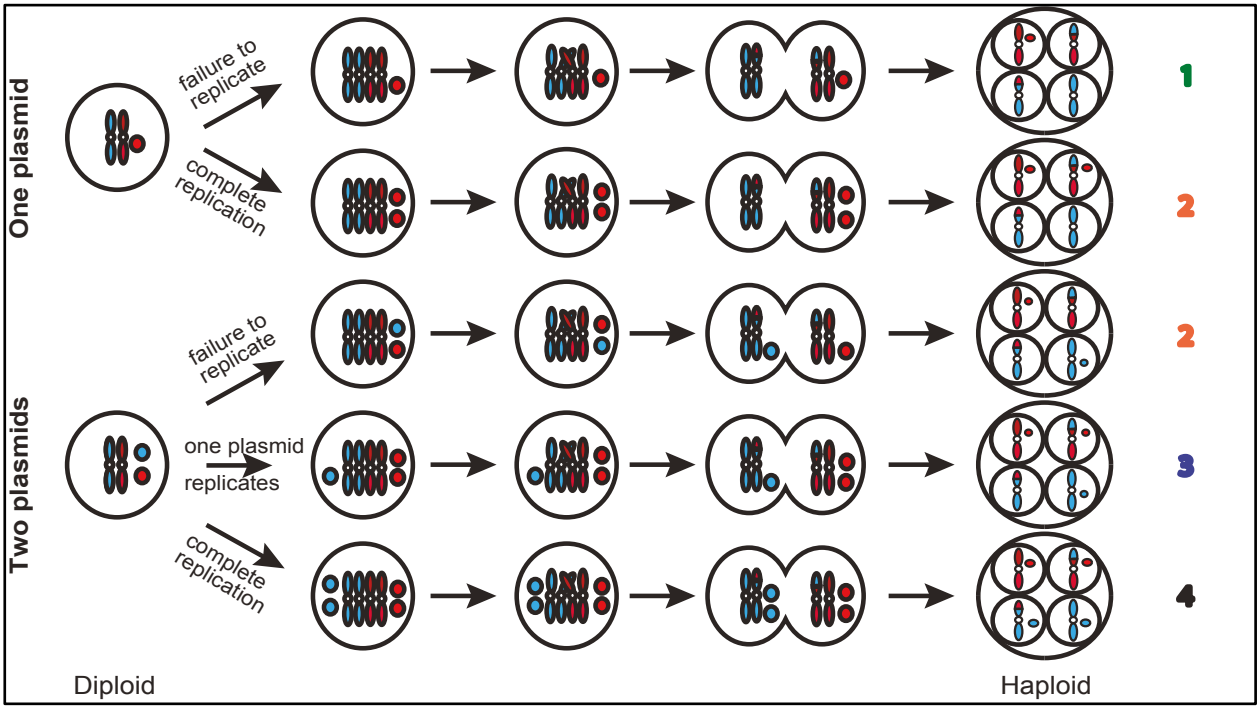


Figure 52: Schematic of additional possibilities for plasmid loss during meiosis

As described in the earlier section where cell acquires a single plasmid, origin activity is indicated by the presence of two viable spores and presence of only one viable spore indicates origin inactivity at the end of meiotic event. The other case is where a cell inherits two plasmids. In this case, three events can be observed, i) where the plasmids fail to replicate: at the end of meiosis, both the plasmids just segregate giving two viable spores. There is no replication events observed in this case. ii) In this case, only the origin on one of the plasmid is active and the other plasmid is inactive. The active plasmid replicates giving two plasmids whereas the inactive plasmid does not replicate. In such case, at the end of meiosis, three viable spores can be observed. iii) The final case is where both the plasmids are active; both replicate and give four viable spores at the end of meiosis. The difference in the presence of two viable spores as a result of a cell with single plasmid replication or a cell with two plasmids not replicating was identified using selectable markers present on different chromosomes. Yeast selectable markers were used to identify the differences between meiosis I and meiosis II. The differences during plasmid inheritance were studied by comparing the inheritance on centromere-linked marker and non-centromere linked marker. The selectable markers used in this study were *HIS3, LEU2, URA3, TRP1, ARG4* and *LYS2* (Figure 53). Selectable markers are present on different chromosomes. The left arm of the chromosome is considered as the shorter arm and right arm to be the longer arm. The distance of the selectable markers on the chromosome is indicated in figure 53 in centimorgan (cM). *TRP1* is

a centromere-linked marker whereas *HIS3, LEU2, LYS2, ARG4* are non-centromere-linked marker.

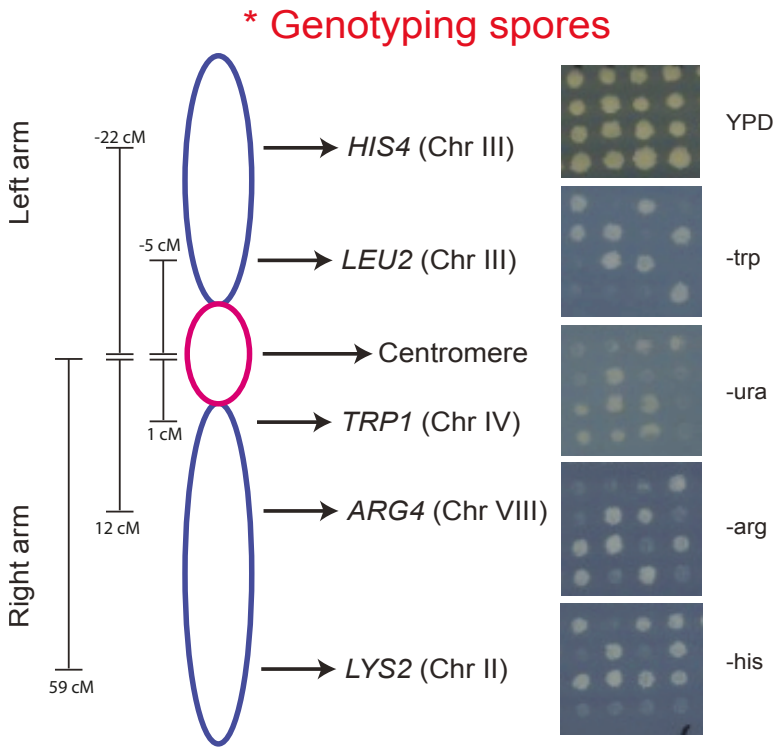


Figure 53: Position of various markers on indicated chromosomes showing their distance from the centromere. Cartoon showing chromosome arms and the location of selectable markers. The figure on the right indicates the spore viability of some sample tetrads on different marker plates.

The difference between chromosome segregation and replication was analyzed by comparing the plasmid inheritance on centromere-linked and non centromere-linked markers. Replication success was confirmed by a 2:2 segregation pattern on a centromere linked marker media and a non-centromere linked marker media. An improper 2:2 segregation pattern indicates replication failure. The pattern for identification was visualized as shown in figure 54. Figure 54 explains the identification pattern to differentiate replication and segregation in the assay. If the plasmid is replicated, similar pattern is observed in spores of centromere-linked and non-centromere linked markers. However, if the plasmid is not replicated and is just a result of two plasmids segregating, the segregation pattern varies between centromere linked and non-centromere linked markers.

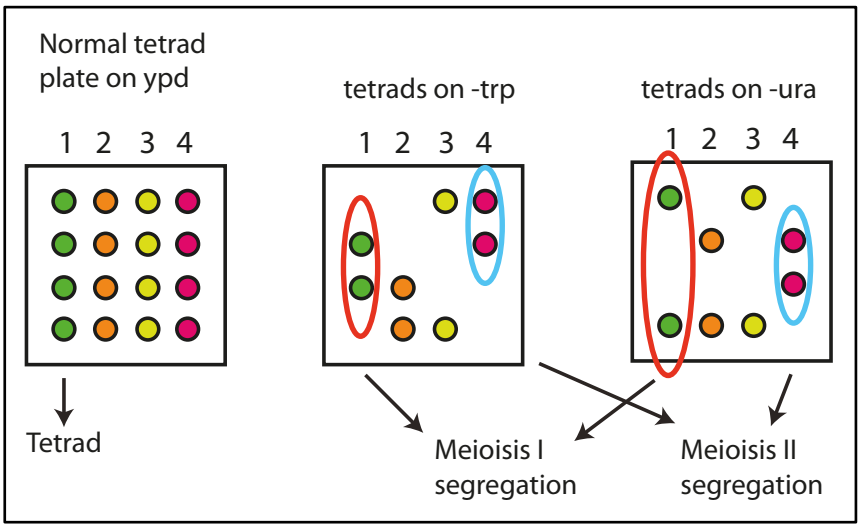


Figure 54: Cartoon showing the events during meiosis I and meiosis II. The cartoon shows the 4 spores from a tetrad and their inheritance on two marker plates, centromere-linked (trp) and –ura media. Proper 2:2 segregation patterns were seen in replication success and improper pattern was seen in case of replication failure.

During the process of meiotic plasmid loss assay, after the cells were allowed to sporulate on sporulation media, growth of tetrads was identified. Sporulating cells were subjected to the action of Zymolyase in dissecting buffer to dissolve the cell wall. Tetrads were dissected and individual spores were allowed to grow on normal YPD media. Once the spores grow, they were transferred to various selective media through replica plating. Plasmid inheritance was studied by looking at the spore viability of the tetrads on selective media. Figure 55 shows an example of how tetrads were tested on different media. The diploid nature of the tetrads was tested on a –trp plate (Figure 55a). *TRP1* is a centromere-linked marker, which shows 2:2 segregation of the spores. The diploid nature of the tetrad was also tested using other markers as previously discussed on –arg and –his media (Figure 55b). Gene conversion events were observed at the *HIS4* locus due to aberrant segregation, giving occasional 3:1 or 1:3 segregation patterns at the *HIS4* locus.

Replication and segregation differences were observed by comparing the pattern of plasmid inheritance/ spore viability on –trp and –ura media. *URA3* is the selectable marker present on the plasmid. Presence of a single viable spore on a –ura plate indicates that the plasmid origin is inactive. Presence of two viable spores was tested for replication and segregation patterns. A similar pattern of 2:2 segregation in both –trp and –ura indicates replication success. A dissimilar pattern of viable spores on –trp and –ura indicates replication failure. Having the data of viable spores from the tetrads on selective media, plasmid origin activity was calculated using the tetrads that gave two viable spores through meiosis II

segregation pattern over the total tetrads. Plasmid origin inactivity was calculated from the tetrads that gave single viable spore over the total number of tetrads.

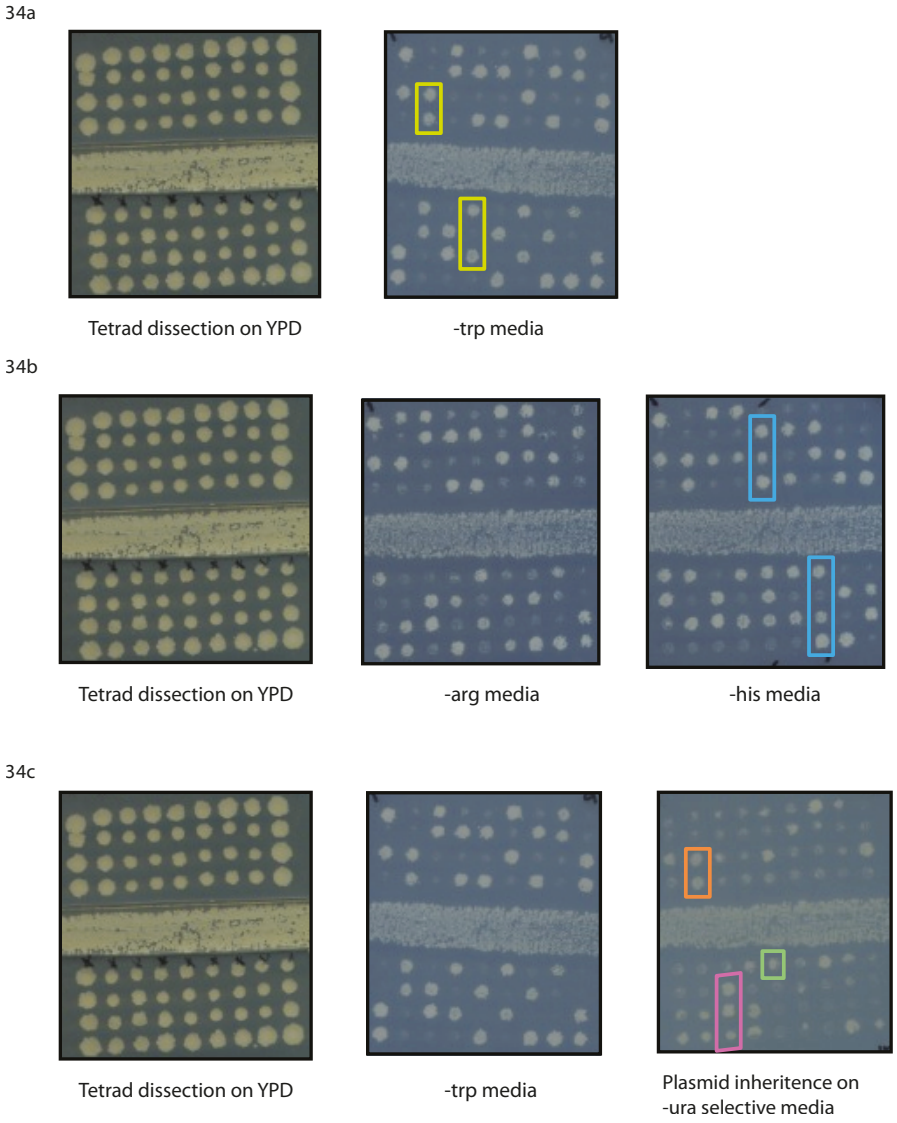


Figure 55a: Diploid confirmation of tetrads on –trp media. Viable spores from YPD plate were replica plated on to –trp to confirm diploid mating type of the tetrads; b: Diploid confirmation on other media, gene conversion events in –his The diploid type of the tetrads was also tested on –arg and -his. Gene conversion events were indicated via 3:1 segregation on –his media; c: Plasmid inheritance on –ura selective media Plasmid inheritance was tested on both –trp and –ura media and compared to analyze the replication and segregation events.

4.5 Differences in origin activity in small vs. larger chromosomal fragments

To find out the mitotic and meiotic activities of selected origins, origin fragments of varying sizes, small and large were cloned and the resulting plasmid were transformed into yeast. The origin activity of each of these plasmids was tested. Initially, the study started with looking at the activity of well-characterized and well-studied intragenic origins. Control origins studied were *ARS416*, *ARS1021* and *ARS607,* all the three origins are well-characterized intergenic origins. For intergenic origins, the larger fragments contain large chromosomal content whereas the smaller fragments contain small chromosomal content.

4.5.1 ARS416

ARS416 is a well-characterized origin and very well studied. *ARS416* was taken as a control intergenic origin to study the mitotic and meiotic activities. *ARS416* slighlty overlaps the TRP1 gene, but the ORC-binding site is intergenic. pCN9 is an *ARS416* containing plasmid with a smaller fragment (400bp) of the origin. pARS1.3 is the larger plasmid carrying 16 kbp fragment of the origin *ARS416.* Figure 56 represents the chromosomal fragments in the two plasmids replicated from *ARS416.*

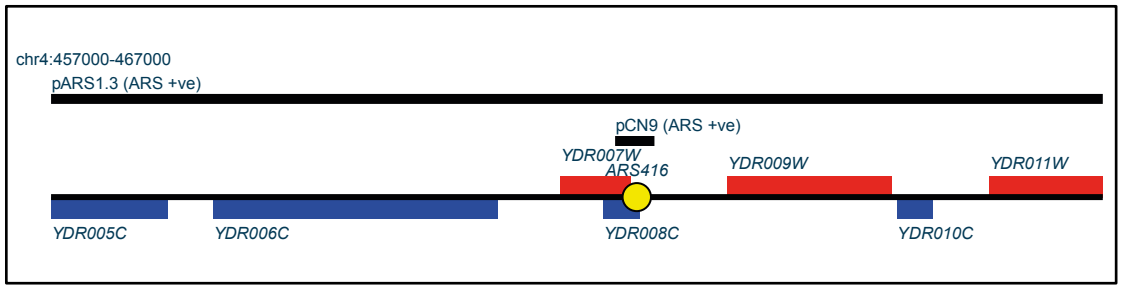


Figure 56: Cartoon showing the small and large chromosomal fragments of *ARS416.* pCN9 and pARS1.3 represent the smaller and larger plasmids of *ARS416*.

The plasmids were tested for mitotic and meiotic activities through plasmid loss assays. The mitotic activity was assayed by calculating the percentage of cells carrying the plasmid over a number of generations. The larger fragment of *ARS416* was found to be highly active, which can be seen in the graph in Figure 57. By contrast, the plasmid containing smaller fragment was lost 6.4%.

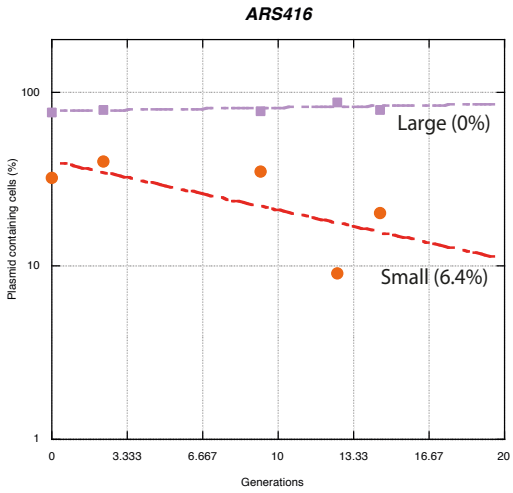


Figure 57: Mitotic activity of control intergenic origin *ARS416* The mitotic activity of larger and smaller plasmids was tested. The larger plasmid was found to be highly active whereas the smaller plasmid was found to be lost to a small extent.

The activity of the origin during meiosis was tested through the meiotic loss assay. The meiotic activity of larger plasmid was assayed to be 94% whereas the smaller plasmid was only 68% active (Figure 58). Similar to the mitotic activity, correlation was observed in the activities of smaller and larger fragments. Both during mitosis and meiosis, smaller plasmids were found to be less active than the larger fragments. This is in accordance with the published data; it was also found in earlier studies that the smaller plasmids have a lower activity than the larger plasmids (Nieduszynski et al., 2005). However, the exact reason for why the smaller plasmids were less active than the larger plasmids is not known. This might be due to the low chromosomal content in smaller plasmids, and/or susceptibility to interfering transcription from flanking plasmid sequence.

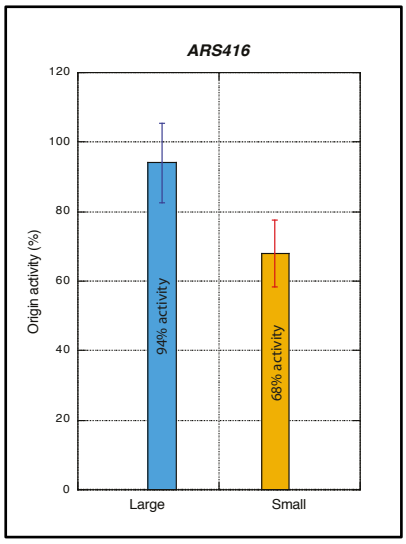


Figure 58: Meiotic activity of control intergenic origin *ARS416* Meiotic activities of smaller and larger plasmids were calculated. Larger plasmid has higher activity than the smaller plasmid during meiosis.

4.5.2 ARS1021

ARS1021 is another well-characterized origin. *ARS1021* was taken as another control intergenic origin to study the mitotic and meiotic activities. pCN10 is an *ARS1021* containing plasmid with a smaller fragment (400bp) of the origin. pARS121.1 is the larger plasmid carrying 12 kb fragment of the origin *ARS1021* (Figure 59a). The plasmids were tested for mitotic and meiotic activities through the loss assays developed. The mitotic activity was assayed by calculating the percentage of cells carrying the plasmid over the number of mitotic generations. The larger fragment of *ARS1021* was found to be highly active, which can be seen in the graph Figure 59. The smaller fragment was lost 6.2%. The activity of the origin during meiosis was tested through the meiotic loss assay. The meiotic origin activity of larger plasmid was assayed to be 90% whereas for the smaller plasmid the origin was only 75% active. Similar to the mitotic activity, correlation was observed in the activities of smaller and larger fragments. In accordance with the previous results and already published data, the smaller plasmid was less active when compared to the larger plasmid.

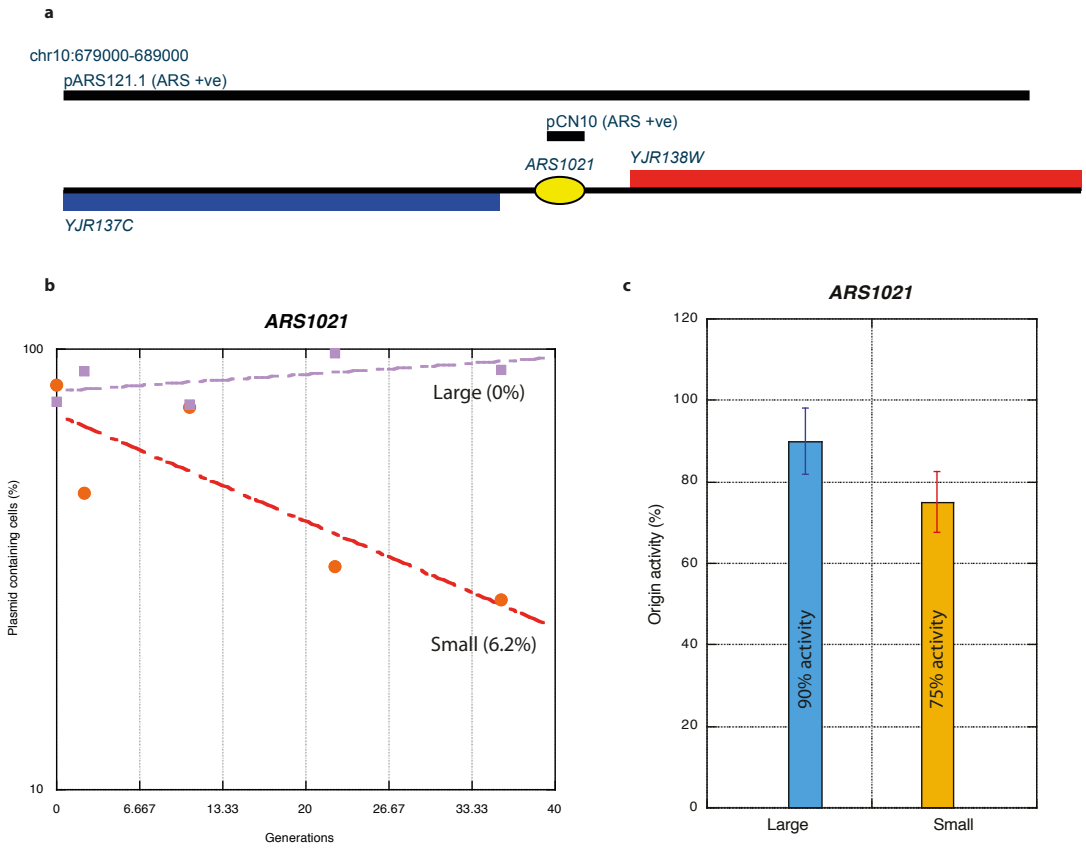


Figure 59a: Schematic showing the two fragments of *ARS1021* cloned. b. Mitotic activity of smaller and larger fragments of *ARS1021*. c. Meiotic activity of smaller and larger fragments of *ARS1021*.

4.5.3 ARS607

ARS607 is another well-characterized origin and very well studied. *ARS607* was taken as a control intergenic origin to study the mitotic and meiotic activities. pCA011 is an *ARS607* containing plasmid with a smaller fragment (400bp) of the origin. pMSH007 is the larger plasmid carrying 6000 bp fragment of the origin *ARS607* (Figure 60a). The plasmids were tested for mitotic and meiotic origin activities through the loss assays developed. The mitotic origin activity was assayed by calculating the percentage of cells carrying the plasmid over the number of generations. There was no observed mitotic plasmid loss for *ARS607* on either smaller or larger fragments. The activity of the origin during meiosis was tested through the meiotic loss assay. The meiotic origin activity of larger plasmid was assayed to be 88% whereas on the smaller plasmid the origin was only 65% active. In accordance with the previous results, the smaller plasmid was less active when compared to the larger plasmid.

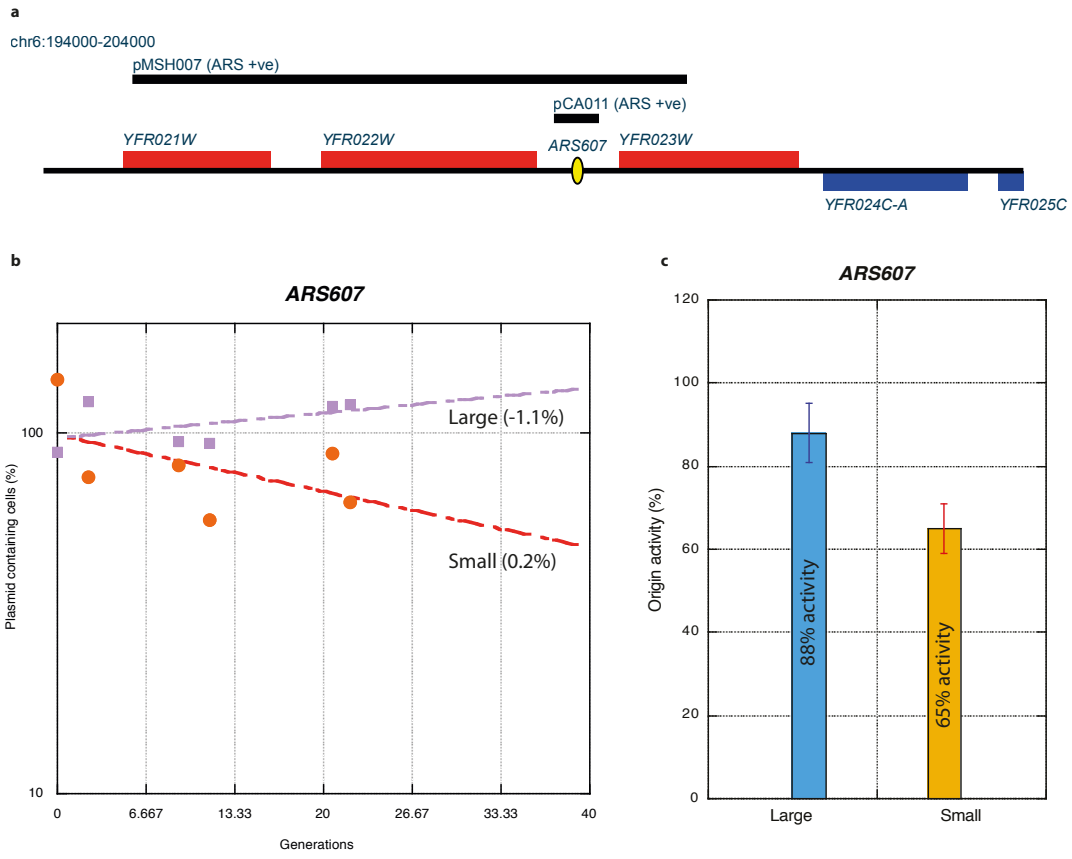


Figure 60a: Schematic showing the two fragments of *ARS607* cloned. b. Mitotic activity of smaller and larger fragments of *ARS607.* c. Meiotic activity of smaller and larger fragments of *ARS607.*

4.6 Lower origin activity of plasmids in meiosis than mitosis

The activity of the three control intragenic origins was compared during mitosis and meiosis. For each of the origin studies, the mitotic activity was compared against the meiotic activity. Interestingly, for all the three origins the meiotic activity was low when compared to the mitotic activity. The examples illustrated in figure 61 were the activities of larger origin fragments. Standard errors were calculated for each of the data collected to look at the statistical significance of the data and the differences were found to be statistically significant. All the data obtained for the mitotic and meiotic activities was plotted for all the plasmids tested (Figure 62). From the plot it is clear that the mitotic activity of all the plasmids is higher when compared to the meiotic activity.

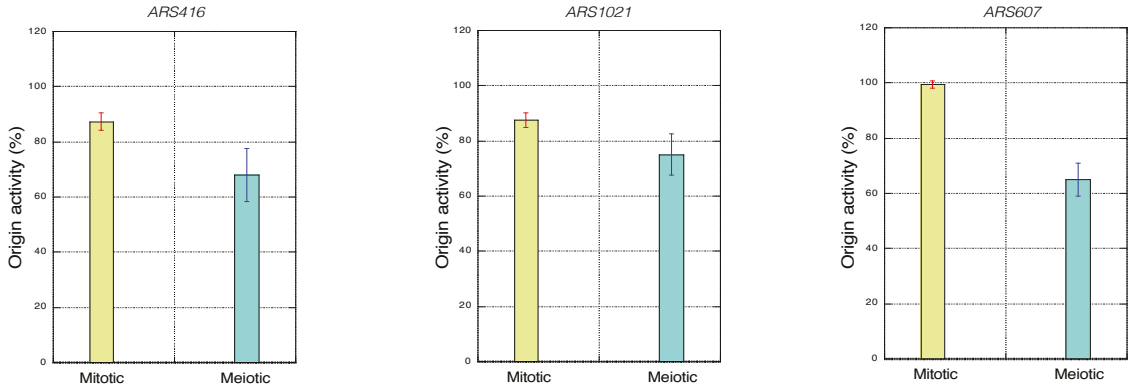


Figure 61: Comparison of mitotic and meiotic activities of control intergenic origins measured on plasmids carrying large chromosomal inserts. The mitotic and meiotic activities of the three control origins, the difference in their activities was found to be statistically significant.

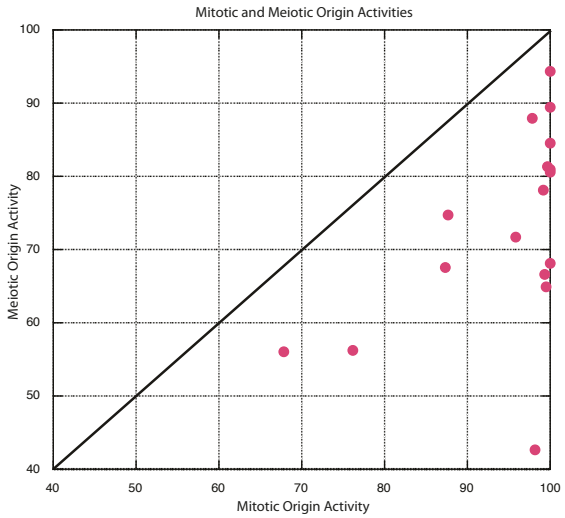


Figure 62: Comparison of mitotic and meiotic activities of all plasmid-based origins. The mitotic and meiotic data for all the plasmids assayed were plotted on the graph, with mitotic activity on X-axis and meiotic activity on Y-axis. The data points clearly indicate the high mitotic activity of all the plasmids when compared to the meiotic activity.

4.7 Intragenic origin activity is consistent with the time of gene expression

Having found that the meiotic activity of the origins was less when compared to the mitotic activity, I then proceeded to study the meiotic activity of origins that were present in genes. The main aim of the work presented here was to study if there is any relation between gene expression and origin activity. Many studies to date have suggested the presence of replication origins in between the genes, which is the most common pattern. It was suggested that the detrimental effect of gene expression might be responsible for the lack of origins in the gene. Meiotic origin activity was mainly studied for the set of origins that were found to be present in genes. The larger plasmids (with the promoter of the gene) and the smaller plasmids (without the promoter region of the gene) were tested.

Initial analysis of origins present on early expressed genes gave some interesting findings. From previous published data and previous data from this study, it was always observed that the smaller plasmids were less active when compared to the larger plasmids. However, for origins *ARS605* and *ARS430.5* the meiotic activity was found to be lower for larger plasmids than the smaller plasmids (Figure 63). Analysis of these origins showed that these two origins are present on genes, *MSH4* and *ZIP1*, which are expressed early during meiosis. The larger plasmids, which have the promoter region on them, lost their activity more than the smaller plasmids that lack the promoter region.

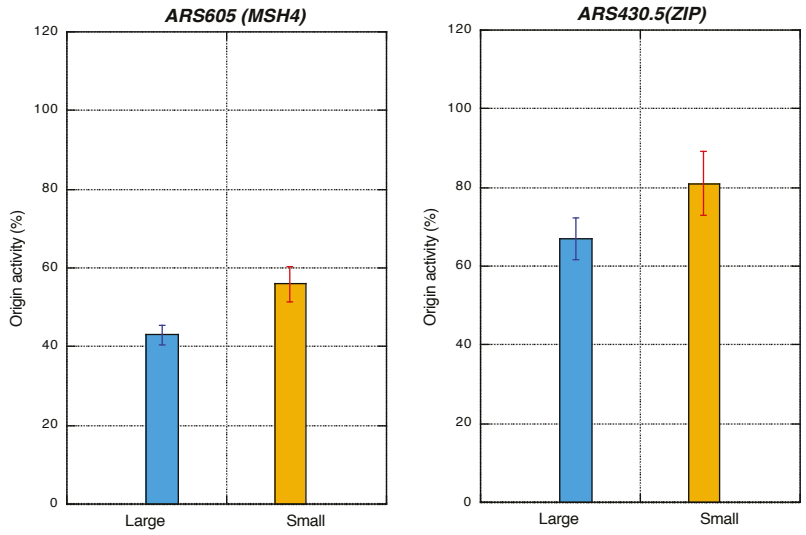


Figure 63: Meiotic activity of origins present in genes expressed in early (before and during DNA replication) meiosis.

The meiotic activity of origins *ARS218* and *ARS427.5* on larger and smaller plasmids was found to be opposite to the activity of the previous intragenic origins (Figure 64). The smaller plasmids were less active when compared to the larger plasmids. The result was not statistically significant for origin *ARS427.5,* however the pattern was consistent.

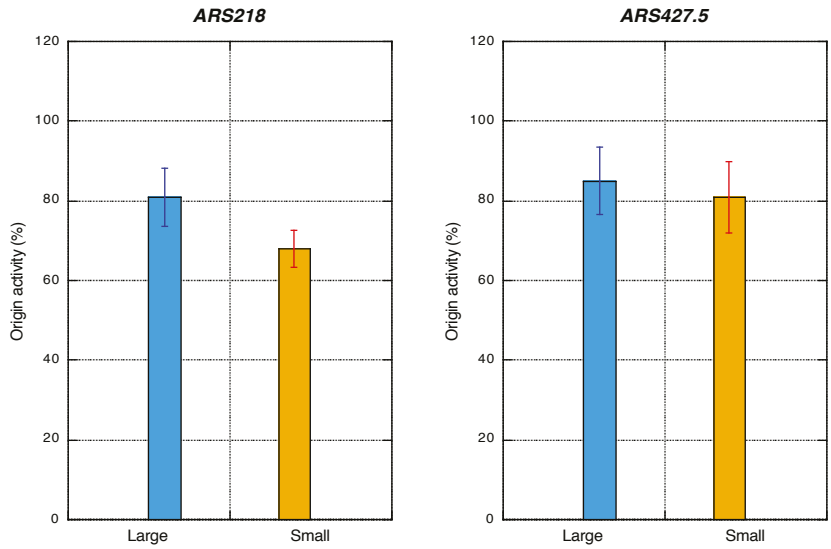


Figure 64: Meiotic activity of origins present in genes expressed after DNA replication

The results of meiotic activities of all the intragenic origins tested were gathered into a graph for comparison (Figure 65). From the figures and data shown below, for the origins *ARS605* and *ARS430.5* the activity of larger plasmids carrying the promoter region of the gene were found to be lost highly when compared to the smaller plasmids. Careful examination of the gene activity showed that these two origins are present on genes *MSH4* and *ZIP1*, which are expressed early during meiosis. The early expression of these genes might be affecting the activity of the larger plasmids carrying the promoter region. The other two origins *ARS218* and *ARS427.5,* where the activity of smaller plasmids is lower than the larger plasmids, these origins are present within genes, which are expressed later during meiosis. This might explain the differences in the relative activities on smaller and larger plasmids of the various origins. The relation between gene expression and origin activity might explain this. The early expression of the gene might prove to be detrimental to subsequent origin activity.

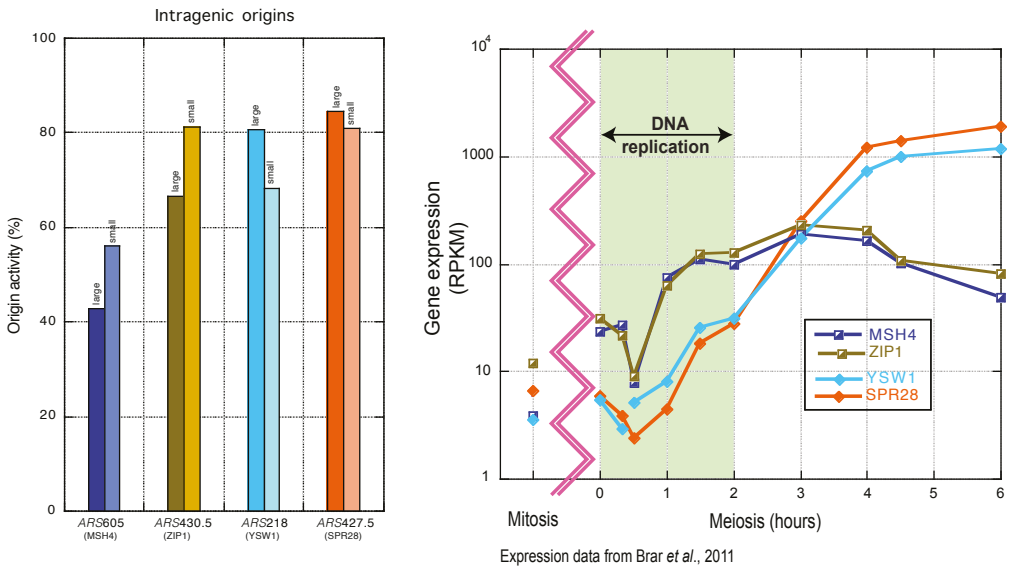


Figure 65: Comparison between origin activity during meiosis and gene expression

4.8 Promoter swaps

From the previous discussed data, it was hypothesized that the presence of promoter region from an early expressed gene on a plasmid might affect the origin activity on that particular plasmid. This can be hypothesized as: gene expression has an effect on origin activity with the early expressed genes having a higher effect than the later expressed genes. To test this hypothesis, I used the concept of promoter exchange. The technique of promoter swap enables us to replace chromosomal promoter sequences with engineered promoters to control the gene expression (McCleary, 2009).

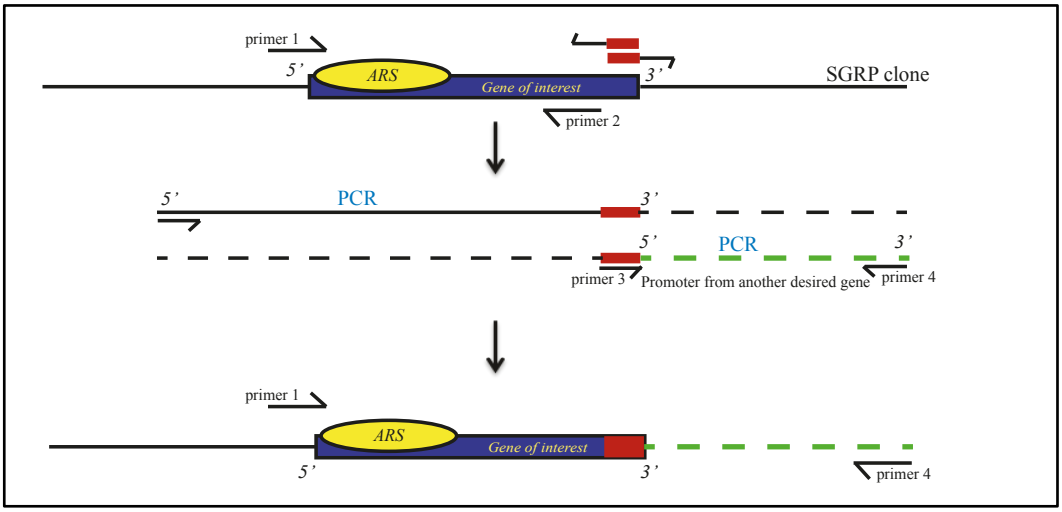


Figure 66: Cartoon showing promoter swap strategy based on PCR-fusions.

As shown in figure 66, an SGRP clone with the gene of interest was selected and the specific genic portion was cloned excluding the promoter region. The promoter from the desired gene was taken out by amplification. The joining primers were designed with regions of homology for amplification. Both the amplified products were fused via fusion PCR. The final product was tested on different selectable markers specific for the gene.

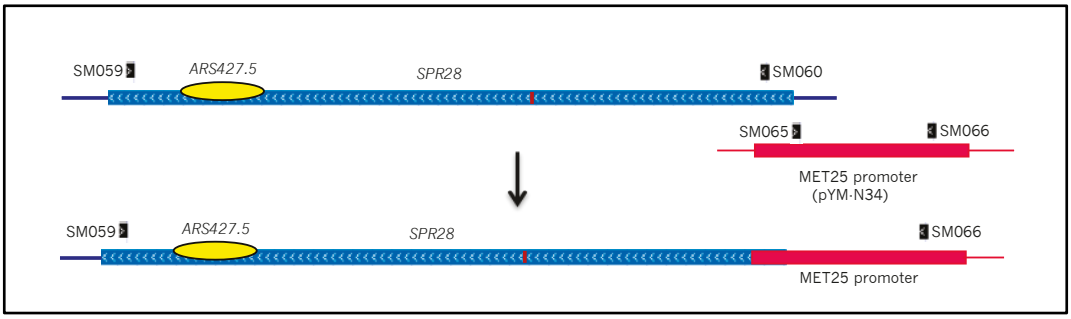


Figure 67: Promoter swap for testing the affect of gene expression on mitotic origin activity

Promoter swaps were done to test both the mitotic and the meiotic activities. To test the mitotic activity, the promoter of a gene on which the origin is present, was swapped with a *MET25* promoter. *MET25* promoter was amplified and taken out from a plasmid pYM-N34. The *MET25* promoter is repressed in the presence of methionine. Under repression conditions, *MET25* messenger RNA is reduced tenfold when compared with non-repression conditions. This indicates that the expression of *MET25* is regulated transcriptionally. In the presence of methionine, the expression of the gene is off and in the absence of methionine gene expression is on. *SPR28* gene carrying the *ARS427.5* origin was amplified by eliminating the promoter region. A single base frame shift mutation was introduced in the gene to interrupt the translation of the gene. The frame shift mutation was introduced to show that if transcription has any affect on origin activity, it is just the transcription that affects the origin activity and not the translation. The *MET25* promoter amplified from the plasmid was fused with the *SPR28* gene lacking the promoter (Figure 67). The resulting plasmid was transformed into yeast cells and tested for mitotic activity in the presence and absence of methionine.

The same promoter swap strategy was used to study the meiotic activity. In my above results, it was shown that the origins present on early expressed genes were lost to a higher extent when compared to those present on later expressed genes. To prove, if the early expression of the gene has an affect on origin activity, promoter swaps were performed, where the promoter of the early expressed gene was swapped with the promoter of the later

expressed gene. The two genes used for this test were *MSH4* and *SPR28*. *MSH4* expresses early during meiosis and *SPR28* expresses late during meiosis. The promoter from the early expressed gene was amplified and taken out which was further cloned into the later expressed gene. Figure 68 shows the promoter swap strategy used to swap the promoter of a later expressed gene with that of an early expressed gene. The resulting swapped plasmid was transformed into yeast cells and further tested for meiotic activity. However, unusually after the meiotic loss experiment there was more number of dead spores seen. The viable colonies that should appear after growing the dissected tetrads on complete media were unusually very low. Due to this, it was not possible to test the origin activity of the swapped plasmid during meiosis. This might be due to the inappropriate expression of meiotic gene that is responsible for the spore inviability.

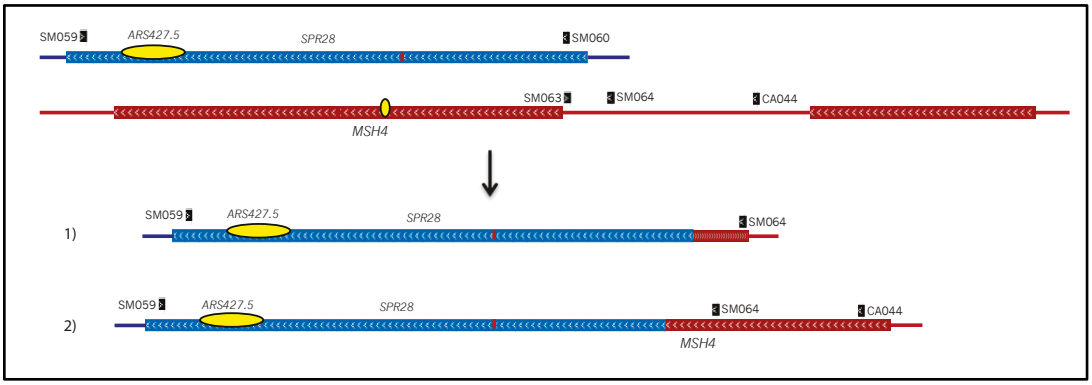


Figure 68: Promoter swap for testing the affect of gene expression on meiotic origin activity

4.9 Studying the affect of transcription on plasmid origin activity through promoter swap

To understand the affect of transcription and origin activity, a hypothesis was proposed as shown in Figure 69. The hypothesis was designed to study the relation between origin activity and transcription. In the first instance, when there is no gene expression, the replication components like the Mcm proteins gets recruited on to the ORC and the origin is licensed, resulting in subsequent replication of the plasmid. In the second case, when the gene expresses early during meiosis, the RNA polymerase shunts off the origin recognition complex (ORC) preventing pre-RC formation and making the origin inactive. In the third instance, when the gene is expressed later during meiosis, by the time the gene is expressed

during meiosis, the process of origin activation is totally completed; hence the process of gene expression has no affect on origin activity.

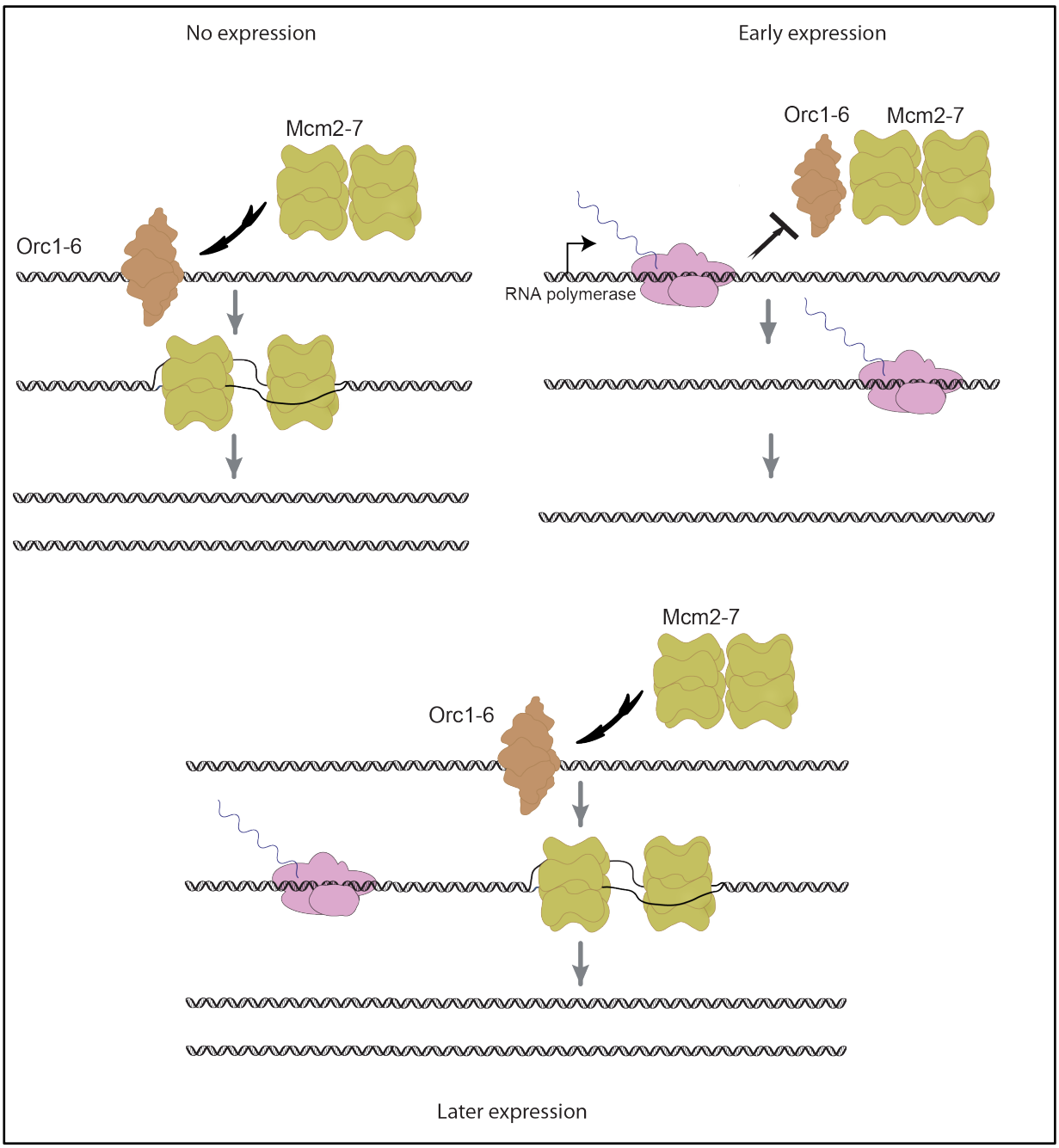


Figure 69: Proposed hypothesis that shows the affect of gene expression on meiotic activity
Schematic showing the three instances of how the origin activity is influenced by gene expression.

The swapped plasmid was tested for origin activity via the meiotic loss assay as described before. However, after the tetrad dissection there were no/ few viable spores seen. Due to this reason the assay couldn't successfully test the relation between transcription and

origin activity during meiosis. This low viability of the spores might to due to some unusual expression of a meiotic gene or a technical error during the promoter swapping experiment, nothing is clear.

4.10 Regulated transcription (MET), influence on origin activity

The plasmid constructed through promoter swap for analyzing the mitotic activity, was transformed into the yeast cells and tested through a specific mitotic loss assay. The whole process of the mitotic loss assay is schematized in Figure 71. The cells were allowed to grow in the presence and absence of methionine, where the *MET25* promoter will be off and on respectively and the origin activity was calculated during those phases. In short, the cells were initially grown in selective media and then transferred to non-selective media. After entering the log phase in non-selective media, the cells were taken in two different environments, one in the presence of methionine and the other in the absence of methionine. Time points were taken at different time points in a similar way as the normal mitotic loss assay. The cells were plated as well in uracil plus and minus media. Mitotic activity was calculated for the plasmids in the presence and absence of methionine and compared.

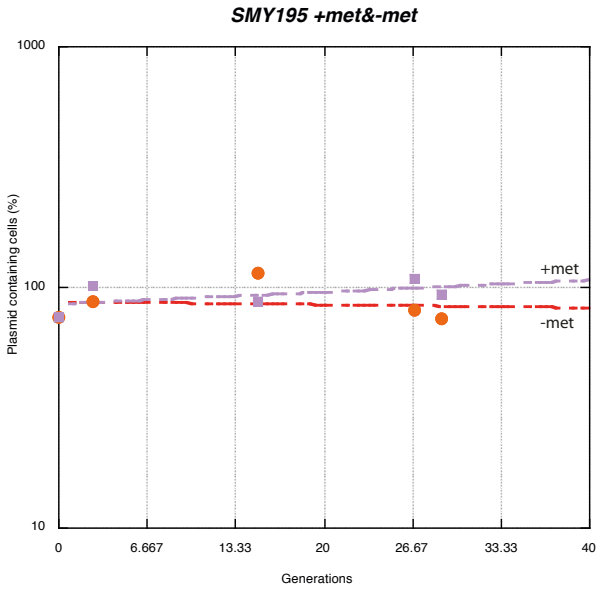


Figure 70: Mitotic activity of MET plasmid in the presence and absence of methionine

The mitotic activity of the swapped plasmid in the presence and absence of methionine was analyzed (Figure 70). From the data obtained and as shown in the graph,

there is no significant difference observed between the activity of plasmids in the presence and absence of methionine. This suggests that the expression of genes has no affect on mitotic origin activity, assuming the promoter construct worked and the gene was transcribed.

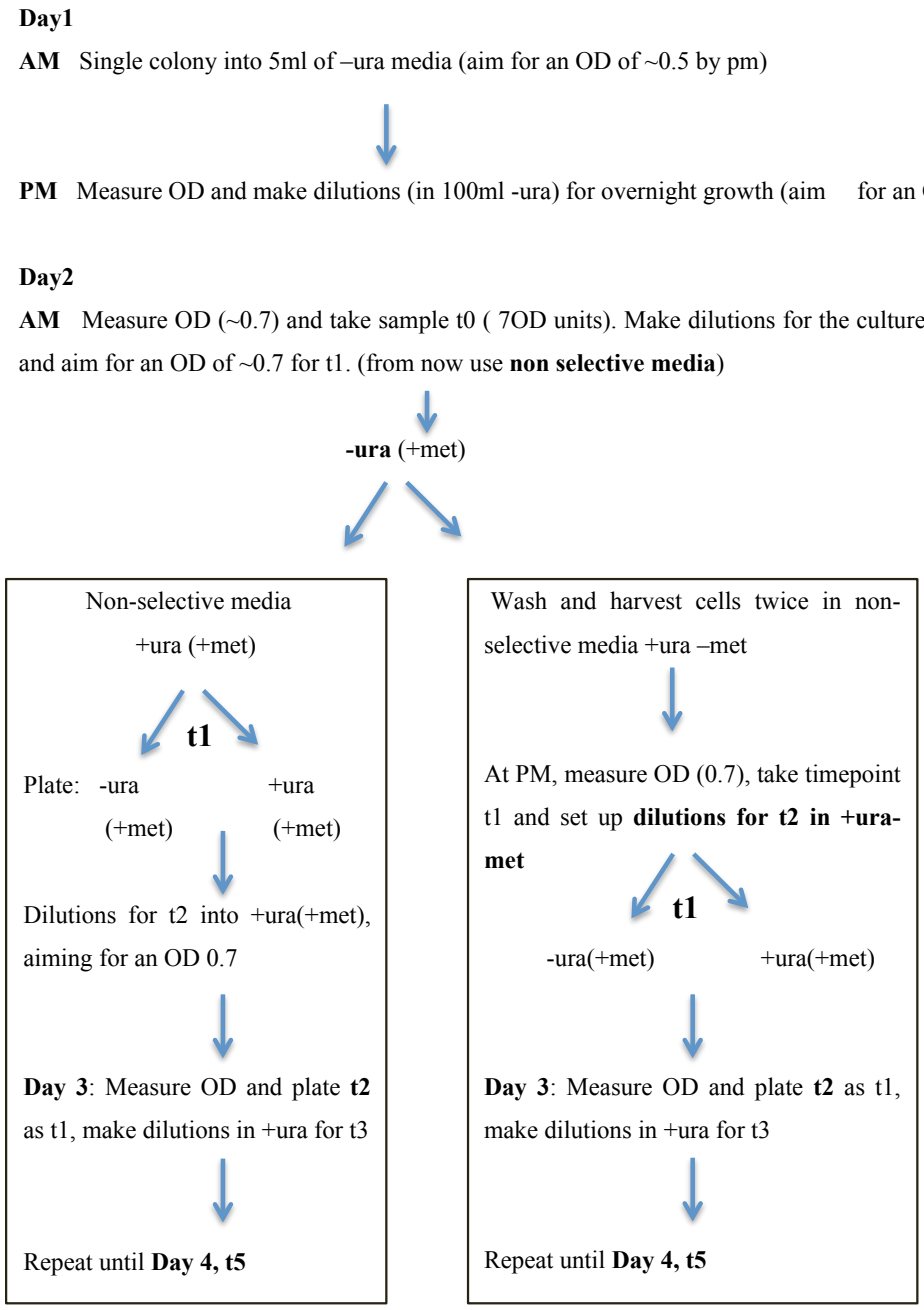


Figure 71: Schematic of transcription regulated mitotic loss assay

4.11 Summary

We started with analysis of developmentally regulated origins. After observation of all the origins, we found that most of the origins are present in between the genes, Intergenic origins. Very few of them were found to be present on the genes, Intragenic origins. Further study found that the few origins which were present on genes, were almost present on genes that have various functionalities during meiosis. To further study the previous assumptions of gene expression being detrimental to origin activity, I have focused on studying the activity of intragenic origins during mitosis and meiosis in both smaller and larger fragments on plasmids. In accordance with the previous studies, the smaller fragments of origins were less active when compared to larger fragments. This was proved earlier during mitosis. In my study, the same was proved during meiosis as well, however with some exceptions. For all the origins and plasmids tested, the meiotic activity of each of the plasmid was always less than the mitotic activity. This might turn out to be one possible reason for why the pre-meiotic S phase is slower than the pre-mitotic S phase.

A further analysis was carried out to study the relation between origin activity and gene expression. It was earlier proposed that the transcription of a gene is detrimental to origin activity (Looke et al., 2010). To prove the hypothesis, I used the strategy of promoter swaps, where the promoters were swapped to change the expression of genes and study the change in origin activity. Promoter swaps were done for both mitotic and meiotic activity, but due to the some problem with the plasmid for meiotic activity, giving all dead spores, I wasn't able to find out the change in meiotic activity after the promoters were swapped. The mitotic activity of the *MET25* swapped promoter was tested in the presence and absence of methionine. No significant difference was seen, indicating that there is probably no affect of gene expression on mitotic origin activity. The exact reason for why there are very few intragenic origins is not clear and yet to be studied. However, the transcription of the promoter swapped was not tested, hence negative results need to be qualified.

5 Discussion

5.1 Regulation of pre-meiotic DNA replication

My study primarily focused on pre-meiotic DNA replication. The mechanisms operating during pre-mitotic DNA replications are well studied, however a mechanistic understanding of pre-meiotic DNA replication is less well studied. It is not known if similar regulation operates during both pre-meiotic and pre-mitotic replications. In budding yeast, mitotic DNA replication initiates at sequence-specific replication origins. Initiation serves as the primary control point for mitotic DNA replication, and is catalyzed by the Cdc7 protein kinase. In contrast, premeiotic DNA replication apparently does not require Cdc7, and the existence and nature of specific replication origins in the meiotic division cycle have not been previously reported (Hollingsworth and Sclafani, 1993). During pre-mitotic S phase, *ctf19Δ* and C-terminal tagging of DBF4 delays centromere replication (Natsume et al., 2013). Delay in centromere replication results in elevated chromosome loss. It is not known whether an equivalent mechanism operates during meiosis. If is the same mechanism operates, then *ctf19Δ* and C-terminus Dbf4 tag is expected to delay centromere replication and elevate chromosome mis-segregation during meiosis. To study this, I used a spore viability assay.

The spore viability assay gives an indirect assay for chromosome mis-segregation during meiosis. To study the role and importance of *DBF4* and *CTF19* during pre-meiotic DNA replication, I constructed homozygous diploid strains with *cft19* deleted and/or Dbf4 tagged at the C-terminus. The spore viability assay data of a *DBF4*-tagged strain was 87% (Figure 7), which is less when compared to the 97% viability of the wild type SK1 strain (Fig 4). This difference was also found to be statistically significant, confirming a difference between the two strains. The reduced viability of a Dbf4-tag strain suggests a possible role of *DBF4* during pre-meiotic DNA replication. The viability of a *ctf19Δ* strain was found to be 0% (Figure 8C). While analyzing the *ctf19Δ* strain, in addition to the tetrads that were tested, most of the tetrads dissected gave dead spores; hence much data couldn't be gathered for the strain. The spore viability assay on these strains showed a role for both *DBF4* and *CTF19* during meiosis, which is consistent with a role during pre-mitotic replication. A further analysis of the strain with double alleles for the genes, *ctf19Δ* and C-terminal Dbf4 tag gave interesting results. The viability of the strain is 17% (Figure 16). Interestingly, *ctf19Δ* strain, which has very low spore viability on its own, regained viability in association with Dbf4 tag.

This seems improbable and the experiment requires repetition to confirm the results. In case it is confirmed, it would be interesting to see how *DBF4* tagging rescues the meiotic function lost due to *ctf19Δ*. At the moment, the exact mechanism/reason responsible for the restoration of viability by Dbf4-tag is not clear. However, spore viability assay offers an indirect assay of measuring effects on replication origins.

DBF4 plays an important role during mitotic DNA replication. The Dbf4 protein interacts with and positively regulates the activity of Cdc7 protein kinase, which is required for entry into S phase in the yeast mitotic cell cycle (Dowell et al., 1994). The role of *DBF4* in pre-meiotic DNA replication is unclear. The data obtained for finding out the role of *DBF4* during pre-meiotic replication seems to be consistent with its role during mitotic DNA replication. It was previously shown that mutants that fail to form double strand breaks (DSBs) showed reduced sporulation and severely reduced spore viability as a consequence of chromosome missegregation (Lam and Keeney, 2015, Keeney and Neale, 2006). The data obtained from my study is consistent with the proposed hypothesis that *DBF4* is essential for faithful chromosome segregation, which is shown by reduced spore viability. However, a more direct assay is required to confirm the role in pre-meiotic DNA replication. Complications might be due to multiple potential roles of *DBF4* during meiosis, including DSB formation. Deletion of origins next to the centromere on Chromosome IX delays centromere replication (Natsume et al., 2013), and such a strain could offer an approach to confirm that centromere replication time is important for spore viability, independent of altering Dbf4 function. To find out the further role of *DBF4*, further assays can be done, such as a replication-timing assay, which can detect changes in replication time, thereby confirming a role for *DBF4* during pre-meiotic DNA replication.

5.2 Measuring genome replication during meiosis

Whole genome replication during meiosis is less understood when compared to mitotic replication where numerous studies have been performed. DNA replication checkpoint prevents entry into mitosis when DNA replication is incomplete, which is crucial for maintaining genome integrity. Very little is known about equivalent controls that operate during meiosis (Murakami and Nurse, 1999). Much progress has been made over the years in understanding meiotic recombination and the two meiotic divisions leading to recombination, however the processes leading up to recombination, including the prolonged pre-meiotic S phase (meiS) and the assembly of meiotic chromosome axes, remain poorly defined. Genome-wide approaches in *Saccharomyces cerevisiae* were used to measure the kinetics of

pre-meiotic DNA replication and to investigate the interdependencies between replication and axis formation (Blitzblau et al., 2012).

To further investigate meiotic DNA replication, I used enriched S phase cells to obtained genome wide replication data. Enriched S phase cells were synchronized and pooled. S phase cells were further enriched by FACS and deep sequenced to obtained a replication profile. As a control to the meiotic replication data, mitotic cells were also FACS enriched from an exponentially growing culture. Data obtained for mitotic cells was of high resolution and the replication profile obtained is similar to earlier published data (Hawkins et al., 2013). The obtained data was compared to the mitotic profiles of various other strains (Figure 20). The replication data of different yeast strains were almost identical apart from a few differences, which are listed in table 3.

I aimed to study the genome wide replication during meiosis. During my work, the Bell lab published work on meiotic replication (Blitzblau et al., 2012). Their study used microarray data analysis, which is of lower resolution than that offered by deep sequencing. The study revealed clear Mcm2-7 peaks at some sites which did not make statistical cutoff. High resolution is very important to study meiosis to allow detection of the (likely) minor differences in replication timing between mitosis and meiosis. To study the meiotic DNA replication, an established genome wide approach (using deep sequencing) was adapted to obtain high-resolution data. In order for the data to be accurate, I have looked for spores during the process of meiotic time course to confirm the proper progression of cells during meiosis (Figure 24). However, the meiotic replication profile data obtained is not of a high-resolution. This might be due to some technical problems during cell recovery or at the cell-lysis stage. A deep sequencing approach is definitely an appropriate way to achieve high-resolution genome wide meiotic replication profile, if the experiment is performed without any technical problems. The problem of poor genome replication data could be overcome in the future by improving synchronization and trying different synchronization approaches, checking lysis of meiotic cells during sample processing, improving ways of extracting DNA, which could be biased by DNA structures including those resulting from meiotic DNA recombination.

5.3 Identification of replication origins within genes

Yeast replication origins are very well studied and most of the origins identified to date were found to be present in between the coding sequences, intergenic origins. It has been

long believed that coding sequences might prove to be detrimental to origin sequences (Looke et al., 2010), hence most of the origins are present only between the genes. However, there is no evidence that can support this statement. To investigate this, I have studied origins present within genes. Careful analysis of genome wide replication data along with our own developed plasmid based assays confirmed that a few origins are present within coding sequences. My aim was to find out if origins are compatible with coding sequences. To study this, I looked at the activity of origin present within genes and how gene transcription influences origin activity.

The mitotic and meiotic activities of origins were assayed via plasmid-based assays (Figure 29). The meiotic activity of all all origins tested was low when compared to their mitotic activity (Figure 41). This might be one possible explanation for why meiotic S phase is longer than mitotic S phase. Interestingly, almost all the origins that are present on genes, called intragenic origins, are present on genes that have meiotic function. The genes on which these origins are present are expressed at different times during meiosis (Figure 43). Some are expressed early during meiosis, while some are expressed late during meiosis. The activity of intragenic origins was studied and their activity compared with respect to their expression during meiosis, to find out if the gene expression time has any affect on origin activity. The affect of gene expression on origin activity was studied during mitosis and meiosis. Activity during mitosis was studied by using a *MET25* promoter (Figure 45). In the presence of methionine, the expression of the gene is off and in the absence of methionine gene expression is on. Origin activity was measured in the presence and absence of methionine when the expression of the gene is off and on respectively. Surprisingly, there was no significant difference observed in origin activities, when the expression of the gene was off and on, consistent with no role for gene expression in mitotic activity (Figure 48).

The relationship between meiotic activity and gene expression was studied by using promoter swaps. The promoter of an early expressed gene was swapped with the promoter of a later expressed gene (Fig 46). I aimed to see if the change in the time of gene expression has any affect on activity of the origins. The origin activity was tested for plasmids after promoter swaps. However for this assay the majority of spores were dead after growing on nutrient rich medium. The exact reason for this is not known, reasons might be due to a technical problem during the assay or might be due to altering the expression time of a meiotic gene. This might also be due to growth conditions, laboratory conditions, however exact reasons are not know. Therefore, it remains interesting to determine the relation between origin activity and gene expression.

5.4 Final Conclusions

The data obtained from this study is consistent with the hypothesis proposed. Starting with finding out the mechanism regulation meiotic and mitotic DNA replication, I have started looking at the activity of *DBF4*. It was earlier shown that *DBF4* plays an important role in mitotic replication. To see its activity during pre-meiotic DNA replication, spore viability assay was adapted. Consistent with our hypothesis, data suggested that *DBF4* plays an important role in pre-meiotic DNA replication, in a similar way as in mitotic replication. However, this needs to be proved further in a more precise way.

Replication origins were studied. Replication origins were mostly found to be present in between genes. This study has proved that there is a subset of origins that lie within genes, which are active both during mitosis and meiosis. This proves that genes are not detrimental to origin sequences. Plasmid loss assays on selected origins showed that the meiotic activity of origins is always low when compared to the mitotic activity. This suggests one explanation for why pre-meiotic S phase is longer than mitotic S phase. Plasmid loss assays were also done on origins to find out the relation between origin activity and transcription. During mitosis, it was found that transcription probably has no affect on origin activity. Further study has to be done to find out the relation between transcription and origin activity during meiosis.

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